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THE BIOLOGICAL BULLETIN

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THE INFLUENCE OF TEMPERATURE UPON THE COMBINATION OF OXYGEN WITH THE BLOOD OF TROUT[1, 2]

LAURENCE IRVING, EDGAR C. BLACK AND VIRGINIA SAFFORD

(From the Edward Martin Biological Laboratory, Swarthmore College, Swarthmore, Pennsylvania)

The air breathed by all species of mammals is the same in composition, and the affinity of their blood for oxygen is also much alike. But the water in which fish live differs greatly in oxygen saturation in different places and seasons, and this variability is particularly conspicuous in bodies of fresh water in temperate regions. Under these circumstances it is not surprising to find that the blood of various species of fish varies greatly in affinity for oxygen. Krogh and Leitch (1919) first remarked upon the differences among the eel, carp, plaice, cod, and trout, and regarded these differences in affinity for oxygen as evidence for the adaptation of the blood to the conditions in which each species lived. But only a few species were examined and these were quite dissimilar in form and habit as well as in respect to the habitat which they occupied.

The catfish, carp, bowfin, and sucker were found by Black (1940) to have blood with different oxygen dissociation curves, which were nevertheless related as if in one family. These freshwater fish are similar in form and are all of free swimming habit. Since their respiratory requirements and their physical systems for providing oxygen are much alike, it is reasonable to consider that large differences in the properties of the blood of these species indicate the suitability of the blood for respiration in different environments.

We have now examined the blood of eight species of freshwater fish and find that the affinity for oxygen varies greatly among the species. Furthermore, as Black (1940) pointed out for four of the species, the

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[1] We wish to acknowledge the kind assistance of Mr. C. O. Hayford, Superintendent of the New Jersey State Fish Hatchery at Hackettstown, in enabling us conveniently to secure the blood from the trout used in these experiments.

[2] The expenses of this investigation were in part provided by a grant from the Rockefeller Foundation.

effect of CO_2 upon oxygen combination is also quite different in the species. Table I shows the usual oxygen affinity and the effect of CO_2 in the blood of these eight species. These characteristics would fit respiration under quite different conditions.

These properties of the blood do not depict its natural suitability for the transport of oxygen because there is no allowance for the variety of temperatures in the natural habitats of the fish. In lakes in temperate regions the temperature of a stratum of water may differ sharply from the temperature above and below, and the seasonal changes are rapid and large. The influence of temperature upon the oxygenation of mammalian blood is such that at 20° it would be 95 per cent saturated with oxygen by a pressure of 45 mm., while at 37° the pressure required is about 100 mm. (Brown and Hill, 1923). According to the figures

TABLE I

Oxygen affinity and effect of CO_2 in blood of freshwater fish at 15°
(Jordan, 1929)

Species	P CO_2 at ½ saturation		Limit of CO_2 effect
	P CO_2=1–2 mm.	P CO_2=10 mm.	Percentage HbO_2
Common catfish *Ameiurus nebulosus*	1.4	5	(Black, 1940)
Bowfin *Amia calva*	4	9	(Black, 1940)
Carp *Carpiodes cyprinus*	5	8	85 (Black, 1940)
Yellow perch *Perca flavescens*			77 (Irving, unpublished)
Common sucker *Catostomus commersonnii*	12	43	71 (Black, 1940)
Chain pickerel *Esox niger*			53 (Irving, unpublished)
Rainbow trout *Trutta iridea*	18	35	52 (Irving, Black, and Safford—this paper)
Brown trout *Trutta trutta*	17	39	52 (Irving, Black, and Safford—this paper)
Brook trout *Salvelinus fontinalis*	17	42	52 (Irving, Black, and Safford—this paper)
Atlantic salmon (freshwater) *Salmo salar*	19	35	57 (Irving, 1939)
Lake trout *Cristivomer namaycush*			40 (Irving, unpublished)

given for the blood of the skate (Dill et al, 1932), the hemoglobin would be 95 per cent saturated by 40 mm. pressure of oxygen at 15°, but the same saturation would require 170 mm. at 25°. At the higher temperature part of the efficacy of the hemoglobin for oxygen transport would be lost even in water which was saturated with air. The blood of the eel would be 95 per cent saturated with oxygen by pressures of 12, 25, and 71 mm. at 5°, 17°, and 30° respectively (Kawamoto, 1929). From general considerations concerning the nature of hemoglobin, as well as from these two examples, it is to be expected that the function of oxygen transport in fish blood is considerably influenced by the temperature at which it occurs.

The combination of oxygen with the blood of fish is peculiarly sensitive to carbon dioxide, as Krogh and Leitch (1919) first observed. Even at 700 mm. pressure of oxygen the hemoglobin of the blood of the tautog will not become saturated with oxygen in the presence of carbon dioxide (Root and Irving, 1940). In this respect the CO_2 effect in fish blood differs from the Bohr effect produced by CO_2 in mammalian blood. In the practical absence of CO_2 the hemoglobin of fish blood is saturated with oxygen at 150 mm. pressure. With increasing pressures of CO_2, oxygen saturation falls off until no further reduction of oxygen saturation is secured beyond 60 mm. pressure of CO_2 (Root, Irving, and Black, 1939). At the limit of the CO_2 effect on the blood of trout only about 50 per cent of the hemoglobin can be oxygenated.

The limit of the CO_2 effect is quite different in various species of fish. A number of freshwater fish which we have examined clearly show the variation in the limit of the CO_2 effect in the blood of several species (Table I, column 3). The differences in CO_2 sensitivity of the blood and the influence which the CO_2 effect would exert upon conditions essential for respiratory transport, however, have only been determined at 15°.

The limit of the CO_2 effect in trout blood changes with temperature, as is shown by the observations recorded in Fig. 1. These determinations were made by measuring the percentage of saturation of the hemoglobin with oxygen at a pressure of 150 mm. and CO_2 at 65 mm. or more. The blood samples were secured from a number of brook, brown, and rainbow trout. The limit of the CO_2 effect falls at a lower level of oxygen saturation with increasing temperature up to 25°. Above 25° the blood cannot be saturated by oxygen at 150 mm. pressure even in the absence of CO_2.

The influence which temperature exerts upon the CO_2 effect shows that the effect of CO_2 upon affinity for O_2 must be considered in making a description of the blood of fish which will be useful in designating its suitability for the performance of its natural function of oxygen transport.

MATERIAL USED

For examining the effect of temperature upon oxygen affinity and the CO_2 effect we have selected three common and related species of freshwater fish; brook trout, *Salvelinus fontinalis;* brown trout, *Trutta trutta;* and rainbow trout, *Trutta iridea.* These fish live under similar conditions and are alike in activity. They were raised in the same water at the New Jersey State Hatchery at Hackettstown. All had received the same sort of food, and each species had been raised from a homogeneous and selected stock. If they were as different in form and habits as the toadfish and the tautog, all of the physical components of the respiratory mechanism would obviously be different, and a comparison of the species on the basis of the properties of blood alone would

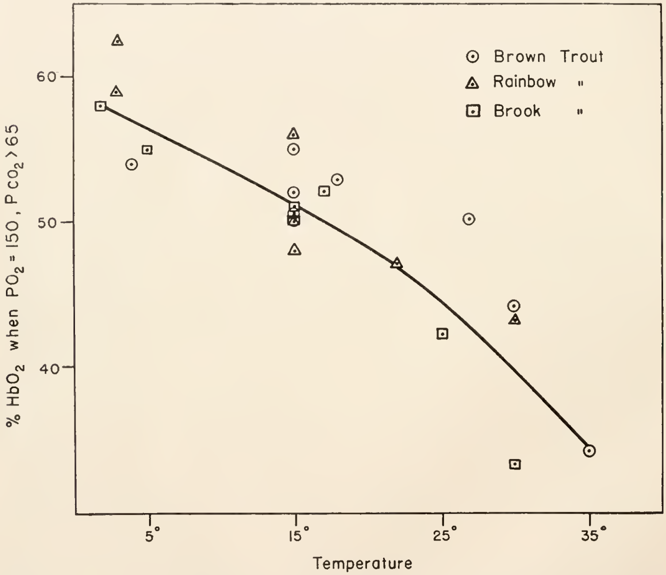

FIG. 1. Limit of the effect of CO_2 upon oxygenation of hemoglobin in trout blood.

not be justified. But since these three species of trout are so much alike, against the background of general similarity of essential respiratory devices either similarity or difference in the properties of the blood for respiratory transport may acquire significance for the eventual picture of specific respiratory adaptation.

Methods

Male fish of from 1½ to 2½ pounds weight were bled by syringe from the heart, yielding from 5 to 20 ml. per fish. Coagulation was prevented by liquid heparin (Connaught Laboratory), the blood was iced, and analysis was begun about five hours after drawing and completed within the next fourteen hours. About five of the eighty fish used died but the others showed no ill effects from bleeding.

The blood was equilibrated for more than 20 minutes with suitable gas mixtures. Half-milliliter samples were withdrawn and analyzed according to the method of Van Slyke and Neill (1924). Extraction of the blood in the apparatus was complete after six minutes. Samples of the equilibrated gases were analyzed in the Haldane apparatus. Hematocrit measurements were made with a high speed (6000 r.p.m.) centrifuge.

Each sample of blood was analyzed after equilibration at from two to five temperatures. If the procedure was prolonged, final results were checked by repetition of an early equilibration. It was possible to keep the blood without change at 30° for at least an hour, but after a short time at 35° the oxygen capacity was not restored by equilibration at 15°.

Mixtures of the blood of several fish gave results comparable with the blood of single fish. Blood samples from 23 brook trout, 23 brown trout, and 34 rainbow trout were examined on five separate days for each species. Each species was examined in March, October, November, and December. No fish were used soon after stripping the sperm for breeding, and there was no apparent seasonal change in the blood.

The temperature of the natural spring water at Hackettstown was uniform at about 12°, but in the pools it fell as low as about 6° without noticeably altering the blood. With a good flow of water in the hatchery and stock selected for a number of years conditions are especially favorable for a degree of uniformity that has not been encountered in our experience with several other species of wild fish.

Oxygen Capacity

When equilibrated with air at temperatures below 25° and with pressure of CO_2 less than 2 mm., the hemoglobin is saturated with oxy-

TABLE II

Oxygen capacity

Species	Number of fish	HbO_2 ml. O_2 per 100 ml. blood	Cell volume ml. O_2 per 100 ml. blood
Brook trout *Salvelinus fontinalis*	23	11.7 (11.0–13.9)	40
Brown trout *Trutta trutta*	23	12.2 (11.1–14.4)	35
Rainbow trout *Trutta iridea*	34	13.8 (13.0–15.0)	43

gen. The oxygen combined with hemoglobin was determined by subtracting from the total oxygen in the blood the dissolved oxygen, which was estimated from the solubility coefficients for oxygen dissolved in mammalian blood (Peters and Van Slyke, 1935). The amount of oxygen combined with the saturated hemoglobin is designated as oxygen capacity. Its value is shown for the three species in Table II. Since the samples were frequently mixed from several fish, the variations in oxygen capacity are not the limits of those which may occur among individuals. The oxygen capacities were slightly larger than in the cat-

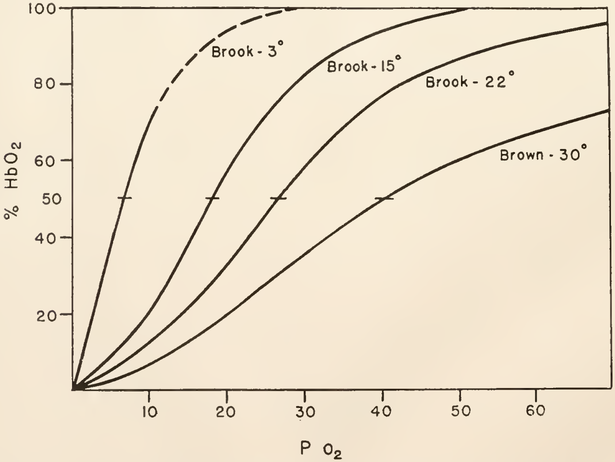

FIG. 2. Oxygen dissociation curves of trout blood at various temperatures.

fish, bowfin, carp, and sucker (Black, 1940), and distinctly larger than in many of the marine fish examined by Root (1931).

OXYGEN DISSOCIATION CURVES

In our experience the oxygen affinity of fish hemoglobin is not perceptibly diminished by CO_2 unless the tension exceeds 2 mm. The oxygen dissociation curves shown in Fig. 2 were made with blood having tensions of CO_2 less than 2 mm., drawing the best lines through points from several samples of mixed blood of the brook trout. One sample

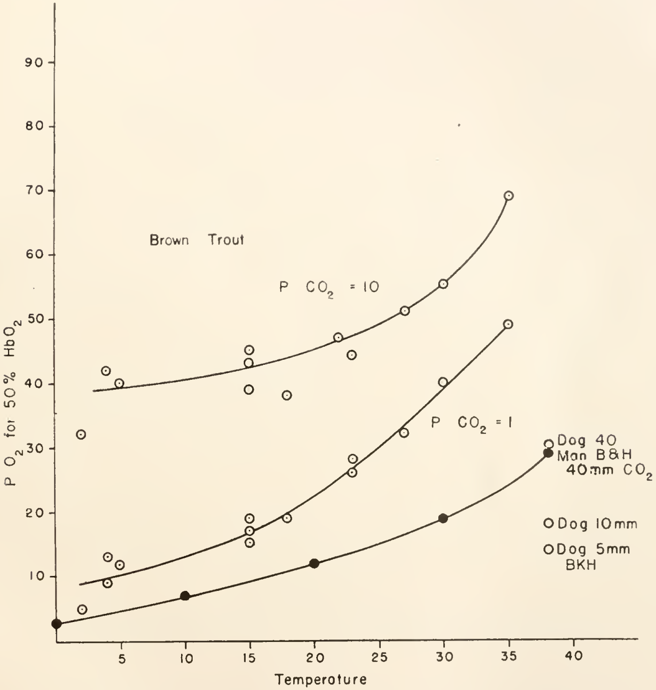

FIG. 3. The effect of temperature upon the pressure of oxygen needed to half-saturate blood of brown trout at CO_2 tensions 1 and 10 mm. Human blood (Brown and Hill, 1923) and some points for dogs' blood (Bohr, Hasselbalch, and Krogh, 1904) are shown for comparison.

of mixed blood from two brown trout was examined over the range of oxygen pressures at 30°. Considering that there are individual variations and that the construction of a number of complete curves for each species at each temperature is not practical, it may be said that these are representative curves, and that one family of similar curves depicts the oxygen affinity of the blood of all three species at various temperatures.

In the gills of trout, loading naturally occurs with tensions of CO_2 in the water which are less than 2 mm. (Ferguson and Black, 1940). In the arterial blood of rainbow trout the tension of CO_2 is about as low, and so the curves represent the condition of the blood when loading with oxygen occurs in the gills. At 30° the blood could not be saturated with the oxygen pressure of the air, but at 25° the hemoglobin could reach its full saturation.

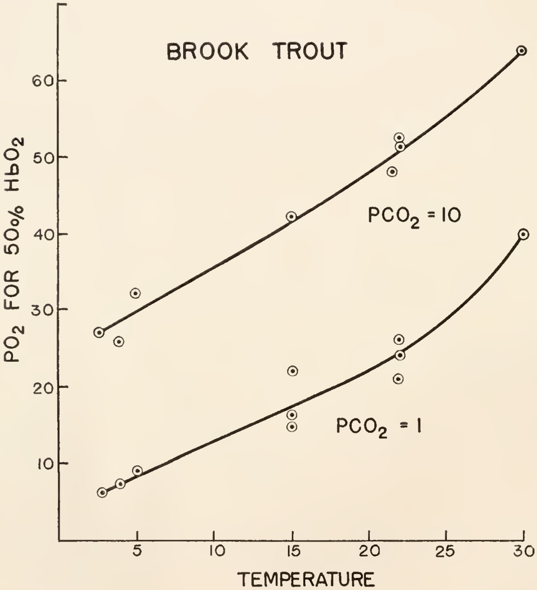

Fig. 4. The effect of temperature upon the pressure of oxygen necessary to half-saturate blood of brook trout at CO_2 tensions 1 and 10 mm.

At temperatures above 15° it is easy to see that the curves are *S*-shaped. At lower temperatures the deviation is too small to be demonstrated with certainty. At any temperature the curve is sufficiently close to a straight line so that a single determination of the oxygen content and tension of blood between 40 per cent and 60 per cent saturation establishes within 2 mm. the point where the curve cuts half saturation. In this manner it was possible by determining the PO_2 for half saturation of the hemoglobin in one lot of blood at a number of temperatures, to estimate the effect of temperature upon oxygen affinity before the blood deteriorated.

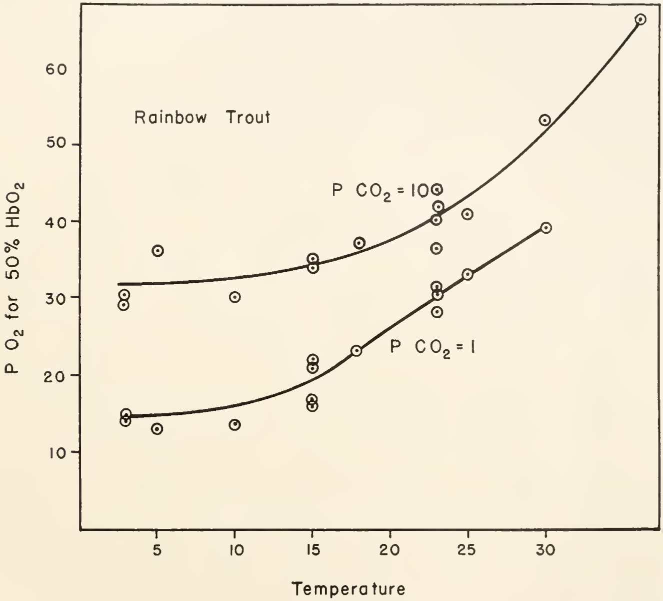

Fig. 5. The effect of temperature upon the pressure of oxygen necessary to half-saturate blood of rainbow trout at CO_2 tensions 1 and 10 mm.

The effect of temperature upon half saturation of hemoglobin in brown trout blood is shown in Fig. 3, the upper curve at 10 mm. CO_2 tension and the next lower at 1 mm. CO_2. For comparison with human blood the lower curve is drawn from the data of Brown and Hill (1923), and points for dog's blood at 37° (Bohr, Hasselbalch, and Krogh, 1904) show how CO_2 affects its affinity for oxygen. Data for the effect of temperature upon blood of brook trout and rainbow trout are shown in Figs. 4 and 5.

When the curves for the three species are superimposed in Fig. 6, it is apparent that without CO_2 there is no difference between the blood of brook and brown trout, but the hemoglobin of rainbow trout requires somewhat greater pressure of oxygen to secure half saturation, particularly at lower temperatures. In the absence of CO_2, the bloods of the three trout are quite similar, but with 10 mm. CO_2 tension significant differences are apparent.

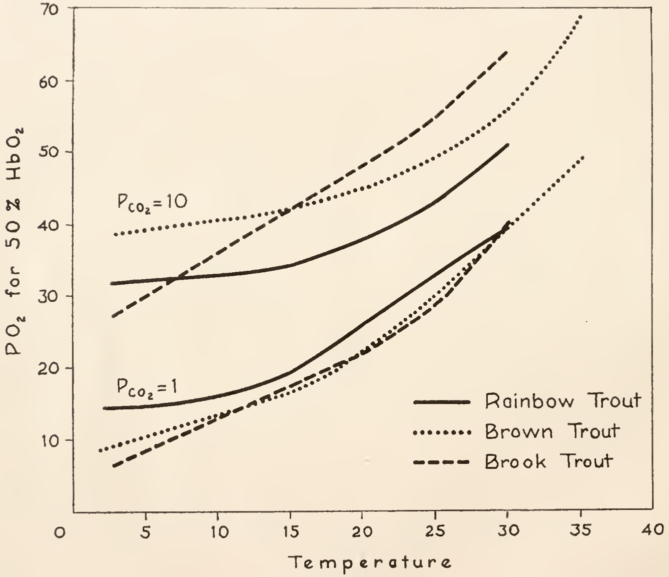

Fig. 6. Comparison of effects of temperature upon oxygen affinity in blood of brown, brook, and rainbow trout.

In the blood of several fish (Root, 1931; Willmer, 1934; Black, 1940), typical oxygen dissociation curves obtained in the absence of CO_2 are shifted to the right by CO_2. The shape of the curve may be altered as well as its position (Root, 1931), and one change in particular appears in the failure of the hemoglobin to become saturated with oxygen even at tensions of 150 mm. As a consequence of this situation the tension of O_2 required for half saturation cannot serve as in the absence of CO_2 to define the whole dissociation curve.

Up to about 65 per cent saturation, however, oxygen dissociation curves in the presence of 10 mm. CO_2 rise approximately straight from the origin. If the oxygen tension for a single degree of saturation between 40 and 60 per cent is determined, that point may then be used to designate the curve as far as the 65 per cent level without appreciable error, and the tension at half saturation locates part of the curve as well as the important physiological condition during unloading of oxygen.

The effect of 10 mm. tension of CO_2 upon the oxygen tension needed for half saturation is shown by the upper curves in Figs. 3, 4, and 5 at each temperature. The distribution of points at any temperature amounted to about 10 mm. in the blood of rainbow and brown trout, but was less in brook trout. The curves do not miss any point by more than 5 mm., and describe the condition of the blood closely, considering the number of samples of blood examined.

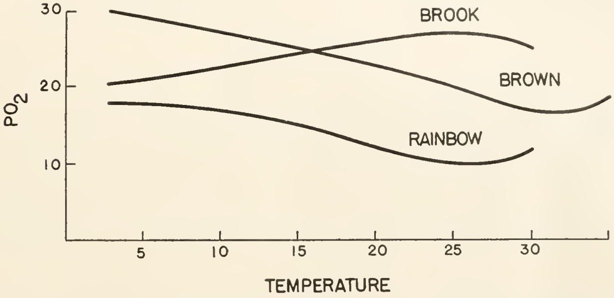

Fig. 7. Increase in PO_2 necessary for half-saturation when PCO_2 is increased from 1 to 10 mm. at different temperatures.

When the curves are superimposed in Fig. 6, it is apparent that at 15° the blood of brook and brown trout are alike with and without CO_2. The effect of CO_2 upon rainbow trout blood is less by nearly 10 mm., which is a greater difference than is shown by individual variations. Over the full range of temperature the curve representing brook trout blood with CO_2 differs from the curve for the other two species in position and slope.

If the addition of CO_2 to the blood occurred in the tissues, the diminished affinity of hemoglobin for oxygen would facilitate the diffusion of oxygen from the blood into the tissues. The situation resembles that

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produced by the Bohr effect in favoring unloading of oxygen from human blood, but in the blood of many fish the influence of CO_2 is much greater. In Fig. 7 there is shown the influence which 10 mm. CO_2 would have in raising the oxygen tension at half saturation and at various temperatures. At 5° the addition of 10 mm. CO_2 requires a rise in oxygen tension for half saturation of 17 mm. in rainbow trout, 20 mm. in brook trout, and 30 mm. in brown trout. The oxygen tension is, however, three times increased by CO_2 in brook trout and only doubled in rainbow trout. The practical bearing of specific differences of this size upon the unloading tensions of oxygen would be important in respiratory transport. It is indicated that the blood of the three species suits its use under different conditions, particularly at temperatures away from 15°, but during unloading in the tissues rather than during loading in the gills.

PROPERTIES OF THE ERYTHROCYTES RELATED TO THE CO_2 EFFECT

Effect of Hemolysis

It is an easy custom to attribute differences among different specific types of blood to specific properties of the hemoglobin involved. But the pure hemoglobin of fish is not known, and there are some striking illustrations that in fish blood the properties of hemoglobin depend upon its condition within the erythrocyte. The effects of CO_2 upon O_2 affinity largely disappear from the blood of the carp and sucker when the blood is hemolyzed (Black and Irving, 1938). The CO_2 effect likewise depends upon the integrity of the erythrocytes in the blood of the tautog and toadfish. On the other hand, the hemolyzed blood of the sea robin still retains part of its sensitivity to CO_2 (Root, Irving, and Black, 1939). The blood of the Atlantic salmon (Irving, 1939) is still sensitive to CO_2 after hemolysis, and so there are instances for the dependence of CO_2 sensitivity upon the erythrocytes in some species and independence in other species.

Samples of normal trout blood and of blood hemolyzed with saponin were equilibrated with 150 mm. pressure of oxygen and several pressures of CO_2 up to about 80 mm., according to the procedure outlined earlier for determining the limit of the CO_2 effect. The CO_2 effect in hemolyzed blood proved to be only slightly less than in whole blood. The limit of the CO_2 effect was about 50 per cent reduction of the hemoglobin in whole blood and about 40 per cent reduction in hemolyzed blood. Evidently the hemoglobin of trout blood, like that of salmon and sea robins, is sensitive to CO_2 whether in the erythrocytes or in hemolyzed blood.

Changes in Cell Volume

We have observed that the erythrocytes of a number of fish swell to a remarkable extent when the CO_2 tension is increased. The erythrocytes of suckers (Black, 1940), tautog, sea robins, and toadfish (unpublished observations), and Atlantic salmon (Irving, 1939), and rainbow trout (Ferguson and Black, 1940) swell considerably, while the cells of carp (Black, 1940) swell very little with CO_2. In the rather small number of species examined swelling is great in those in which the CO_2 effect is large, and small where the CO_2 effect is small.

Comparing the erythrocyte volume of the blood of the trout when the blood was equilibrated with air, with the erythrocyte volume when the same blood was equilibrated with 10 mm. CO_2 and about half saturated with oxygen showed that CO_2 caused swelling in every case. These volume changes are shown in Table III as the increase in percentage over the original volume. Removal of the CO_2 reduced the volume of the erythrocytes again. The swelling of fish erythrocytes with CO_2 is more variable than would be expected if CO_2 tension is the only variable factor which determines volume changes, and erythrocyte volume is certainly labile toward factors or conditions other than CO_2 tension alone.

TABLE III

Increase in volume of erythrocytes produced by 10 mm. CO_2

Species	Number	Range of swelling	Average of swelling
		per cent	*per cent*
Brown trout *Trutta trutta*	14	1–21	10
Rainbow trout *Trutta iridea*	25	4–27	15
Brook trout *Salvelinus fontinalis*	10	1–24	9

CO_2 DISSOCIATION CURVES

The data from the determinations of CO_2 effects were plotted logarithmically as content against tension of CO_2. In all of the blood samples considered the hemoglobin was about half saturated. The points were uniformly distributed, and the regularity justified the construction of the average curves shown in Fig. 8. The points show the mean position of the results obtained with each species, and indicate that there was no difference between the average blood of brook and brown trout.

Blood of rainbow trout has, however, consistently a somewhat smaller CO_2-combining power than the blood of the others. Judging from the slope of the curves, the buffering of the blood of the three species is similar at CO_2 tensions greater than 10 mm. Up to 10 mm. the buffering of brook and brown trout somewhat exceeds that of rainbow trout, although the concentration of hemoglobin in the latter is usually larger than in the other two species.

The amount of CO_2 combined with blood increases with diminishing temperature more rapidly than does the solubility of CO_2 alone. If the CO_2 added to the blood increases the anion concentration, base must be removed by CO_2 from the protein buffers, which, as weak acids, must then undergo a decline in strength in comparison with carbonic acid. The amount of base lost by proteins and gained by CO_2 is equivalent to the change from 26.0 to 36.0 volumes per cent of combined CO_2 between 25° and 3° or 4.5 millimols per liter. Along with this decline in base-binding power of the protein, the affinity of the hemoglobin for oxygen increases as the temperature declines.

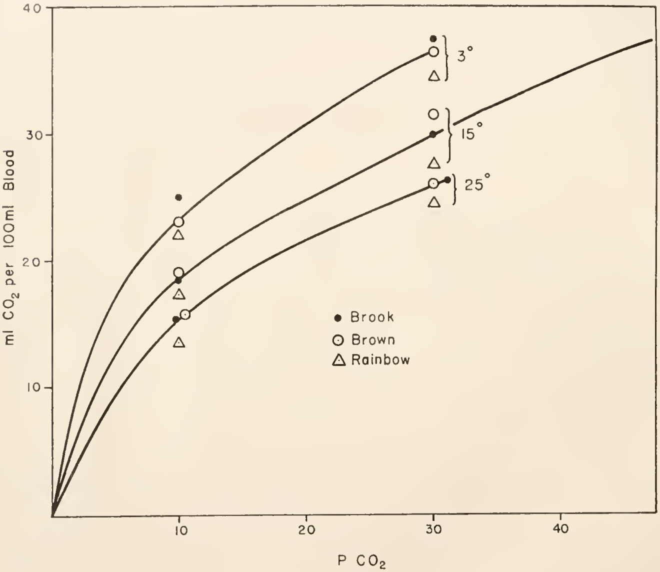

FIG. 8. CO_2 dissociation curves of trout blood at 3°, 15°, and 25°.

Increasing the temperature diminishes the affinity of the hemoglobin for oxygen and increases the ability of the hemoglobin to bind base. The affinity of hemoglobin for oxygen may also be diminished by acidification, but with an opposite effect upon ability to bind base. From these contrasting relations between the effect of temperature and acidity upon oxygen affinity it appears likely that change of temperature does not alter the affinity of hemoglobin for oxygen by affecting its ionization.

Discussion

The affinity for oxygen of the blood of the three species of trout is scarcely distinguishable at 15° and in the absence of CO_2. In the arterial blood of trout the tension of CO_2 is probably only one or two millimeters (Ferguson and Black, 1940), and complete oxygenation in efficient gills would be equally secured for all three species by atmospheric tensions of oxygen in water cooler than 25°. At 20° half of the atmospheric tension of oxygen would suffice to saturate the blood, and it seems likely that under common natural conditions of temperature and oxygen supply the blood of all three species would be sufficiently and about equally oxygenated.

But when the effects of CO_2 and changing temperature are considered, differences appear which distinguish each species. At 5° the normal tension of CO_2, which is about 10 mm. in venous blood, would yield, at half saturation, as unloading pressure, in brook trout 29 mm., in rainbow trout 32 mm., and in brown trout 39 mm. At 25°, the pressures would be 54 mm. in brook trout, 47 mm. in brown trout, and 39 in rainbow trout. The condition of half saturation and with 10 mm. tension of CO_2 represents the situation in the blood when O_2 is passing into the tissues. At low temperature the tension of O_2 available for unloading would be greatest in brown trout, while the blood of brook trout would offer greater unloading tension at higher temperature. The characteristics of unloading are distinct for each species at all temperatures except at 15°, and the change in unloading conditions with temperature is likewise distinct in the blood of each species. The natural consequence would afford to the brown .trout an unloading tension greater than that of the other three at low temperatures. At high temperature the brook trout would have the advantage of greater unloading tension.

The specific differences which have been shown might be attributed to the possession of hemoglobin of a different type by each species, but on this point there is no evidence. Another view could regard the differences as based upon the influence of the milieu upon the oxygen affinity of a type of hemoglobin common to the three species. Hemoglobin is extremely sensitive to the acidity and salts in which it exists, and in natural conditions the slope of the CO_2 curve in trout blood is very steep. The changes in acidity within the erythrocyte may be quite rapid, and that the osmotic changes are apparently quite large is indicated by the considerable swelling of erythrocytes produced by a 10 mm. increase of CO_2 tension. We have not seen any specific difference in the erythrocytes which would affect oxygen affinity, but the differences which we have shown are small, and our observations upon the lability of the erythrocytes are gross. The influence of temperature is undoubtedly exerted directly upon the hemoglobin, but temperature probably influences the properties of the erythrocytes as well, and so indirectly affects the hemoglobin by altering its milieu. The lability of the milieu and the sensitivity of hemoglobin are such that the respiratory functions of quite similar hemoglobins might be greatly modified by the milieu.

Summary

Various species of fish possess blood with different characteristic ability for combining with oxygen. These differences appear to fit the blood of each species for the transport of oxygen under special conditions.

In three closely related species of trout the characteristics of oxygen combination are similar at the low CO_2 tension characteristic of arterial blood. The effect of rising temperature upon the combination of oxygen with the blood *in vitro* of *Salvelinus fontinalis, Trutta trutta,* and *Trutta iridea* is to diminish the oxygen affinity. At 15° their blood is half saturated at 17, 17, and 18 mm. tension of oxygen respectively, and changing temperature increases the oxygen tension required for half saturation about 1 mm. per degree. This situation prevails when the tension of CO_2 is about 1 mm., and only at lower temperature does the blood of rainbow trout become distinguishable from the other two in requiring slightly greater oxygen tension for half saturation.

CO_2 greatly decreases the affinity of the hemoglobin for oxygen. The limit of the effect of CO_2 is reached at about 60 mm., and at that tension at 15° the hemoglobin is only half saturated. Raising the temperature diminishes the degree of oxygen saturation in the presence of CO_2.

When the CO_2 tension is 10 mm., half saturation with oxygen requires about twice the tension of oxygen needed in the absence of CO_2. The curves representing change in oxygen affinity with temperature when the CO_2 tension is 10 mm. are different in position or slope for each of the three species. The differences are large enough to fit the blood of each species for oxygen transport under different conditions.

The erythrocytes of trout blood may swell 25 per cent when the CO_2 tension is increased from one to 10 mm. The swelling is observed in the blood of several species of fish having hemoglobin which is sensitive to CO_2. The CO_2 dissociation curves of the three trout are essentially alike and vary in the same manner with temperature.

The difference observed in the blood of these three species would apparently provide different conditions for unloading oxygen in the tissues, and the change of unloading conditions with temperature is peculiar to each species of trout. Only at temperatures above 20° would aeration at the gills normally be restricted.

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ON THE METHOD OF FEEDING OF FOUR PELECYPODS

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Introduction

The feeding tracts of many pelecypods have been described, but while many of these accounts have approximated the true conditions, there is one important fact that has been omitted, and it is upon this that an understanding of their feeding methods depends.

When the pelecypods used in this experiment were feeding, a sheet of mucus entirely covered the gill structure, and it is this mucous sheet which strains out food material from the water vascular current. Cilia serve only to create the current and move the mucus. With the exception of highly specialized or quite primitive forms, it is probable that this method of feeding is general in the class Pelecypoda.

Except in cases where the animal has been left sufficiently long (usually long enough to begin the regeneration of the shell and mantle), a pelecypod which has been cut open is not feeding. When disturbed, the pelecypods that I have investigated cease feeding at once, and, when brought into the laboratory, several days may elapse before they will feed naturally. Therefore, it is necessary to make certain that the animal which is being investigated is adjusted to its surroundings and is carrying on its activities exactly as though it were in its natural habitat. Many pelecypods will begin feeding shortly after being brought into the laboratory, provided they are not mutilated in any way. In such cases feeding may begin within a few hours, and, in time, these animals actually become somewhat adapted to oft repeated disturbances.

Materials and Methods

Four pelecypods were used, namely, the gaper clam, *Schizothaerus nuttallii;* the mud flat scallop, *Pecten circularis;* the native West Coast oyster, *Ostrea lurida;* and the West Coast mussel, *Mytilus californianus.* These represent a burowing form, a surface form, an above surface form, and an open coast form. The first three use detritus for food, and the fourth uses plankton.

A hole was bored in different positions in one side of the shell of different individuals of each species, so that ultimately all regions connected with the feeding activities of each of these pelecypods could be observed. These windows were covered with a thin piece of glass, the size of the window depending upon what region and how much area of the region was to be investigated.

The openings were made by grinding a portion of the shell away, removing the underlying mantle, and then cementing a piece of cover-glass over the hole. The cover-glass was cut to shape by means of carborundum points and cemented in place with United Mender. Many cements were used, but the United Mender made by the United Sales Co. of New York, Dallas and Los Angeles proved to be by far the best; for windows cemented in place with it remained in place for as long as six months, although they were continuously immersed in ocean water. Before the cement was applied both the cover-glass and the rim of the shell around the opening were wiped clean with a clean cloth dampened with 95 per cent alcohol.

It is necessary to have the shell surrounding the opening perfectly flat before the cover-glass is cemented in place. A sander grinds faster and generates less heat than an emery wheel. While one is grinding the opening the animal should be dipped often into ocean water. A small hand rotor and dentist's drills can be used to advantage to cut the opening, and then only the surface surrounding the opening need be sanded flat.

A binocular and microscope lamp were suitably mounted next the aquarium in such a manner that they could be adjusted to any position.

After a window was put in a shell the animal was left undisturbed in running sea water for about two weeks, then watched carefully through the windows with the binocular to determine if feeding was being carried on in a natural manner. This was determined by introducing into the water some non-irritating material which constitutes the natural food of the animal.

The food material used was either a diatom culture or detritus, the latter being preferable. When the surface of the mud of an estuary or of the ocean is disturbed, a grayish turbidity results. The material causing this turbidity consists of decaying organic matter which is rich in bacteria, protozoa and other organisms such as rhabdocoeles, nematode worms, larvae of many species of marine animals, and, in addition, on mud flats there are usually surface diatoms and single-celled algae. This surface sediment or detritus, which constitutes the main, or, in many cases, the only source of food for burrowing or surface pelecypods, is obtainable in any quantity and is readily eaten by most pelecypods. When introduced as food it does not disturb the feeding activities of the clams. (A diatom culture was found to be better for *Mytilus californianus.*)

FEEDING

In the four species listed above, when feeding begins either mucus is secreted at the upper edges of the gills and is carried in a sheet by the frontal cilia to the free edges of the gills, or it is secreted more or less uniformly over the entire surface. It is then carried in strings along the edges of the gills to the labial palps. The palps perform a selective function, at least to the extent of partially removing undesirable particles, while allowing the rest of the material to pass intact with the strings of mucus directly into the esophagus in the form of food-laden strings of mucus. The main point to be stressed here is that the sheet of mucus covers the entire gill in much the same manner as described for tunicates (MacGinitie, 1939), and intercepts all particles from the current of water which passes through the gills and out through the dorsal or excurrent channels.

I consider a pelecypod to be feeding when a sheet of mucus entirely covers the gills, at which times all particles in the water, however small they may be, are strained out by the mucus. I am referring to undisturbed animals. Mucus may be made to flow copiously from any portion of a gill by direct stimulation, but it is very difficult to determine just where and when the secretion of mucus for feeding takes place. For this reason it took more than two years of careful observation to be sure of the main points set forth in this paper. Since mucus itself is perfectly transparent, the presence of the sheet of mucus is shown only by the included detritus. When feeding is actually going on, as witnessed through a window in the shell, the sheet of mucus may not follow the grooves, but, as shown by particles in it, may be deflected somewhat in an anterior direction, the particles carried by it crossing over the grooves, for they are carried by the mucus and not by the cilia. For example, when the mucus sheet is present in *Mytilus californianus,* the pull of the cilia of the free edge of the gills causes the sheet to be deflected ahead, particularly near the lower edge of the gills. Thus it is seen that the sheet of mucus, and not the cilia, carries the food particles.

When particles are moved by the cilia in the absence of the mucous sheet, as in the case of an opened clam when one valve and mantle have been removed, such particles follow the grooves. As it is the frontal cilia which move the mucus, it is to be expected that when the mucous sheet is absent, that is, when feeding is not taking place, the particles being moved to the free edge of the gills will move parallel to the grooves. The current of water created by the lateral cilia bordering the grooves tends to hold the particles in the grooves as they are being moved by the frontal cilia towards the free edges of the gills. In some pelecypods particles will move both down the ridges and up the grooves. The ciliary mechanisms have been worked out in great detail by Atkins (1936, 1937, 1938).

When a small amount of carmine powder is mixed with the detritus and introduced with the incurrent water, it is usually ingested. In *Mytilus californianus* and *Schizothaerus nuttallii* if carmine alone is introduced in the same manner, some of it will find lodgment on the sheet of mucus already formed; but the sheet nearly always will be cut off at the upper edge and the carmine which thereafter collects on the gills will be carried down the grooves, thence forward along the edge of the gill, and then dropped by the labial palps into the anterior portion of the mantle cavity. In *Mytilus californianus* rejected material that is dropped into the anterior portion of the mantle cavity is carried posteriorly by grooves just within the mantle edge and issues from the posterior end in a continuous string termed pseudofeces. I have carefully observed, through windows at the anterior portion of several *Mytilus californianus,* the rejection of the undesirable material by the labial palps. The palps spread apart and assume a transverse position. The mucous threads from the edges of the gills travel directly to the bases of the ventral palps, down their anterior edges and thence into the ventral grooves of the mantle edge, where the laden threads of mucus travel to the posterior end and out as the pseudofeces mentioned above. When *Mytilus californianus* is feeding, the palps are laid backward outside of and close to the gills.

In *Schizothaerus nuttallii* rejected material will be forcibly ejected from the mantle cavity by a sharp contraction of the adductor muscles, which quickly brings the valves together and squirts the water and rejected material from the mantle cavity out through the incurrent siphon or opening.

This activity undoubtedly accounts for some of the squirting by clams on mud flats when the tide is going out. As the tide is leaving the mud flats, clams that have long siphons and burrow deeply squirt much more often than they do when the tide is in and they are covered by water. When the mud flats are nearly bare a considerable amount of sand and other undesirable material stirred up by the action of the waves may cause material that will be rejected to accumulate rather rapidly. Long-necked clams usually eject water much more forcibly than do those clams which live much nearer the surface. *Schizothaerus nuttallii* squirts water to a height of from 3 to 5 feet. Such removal of rejected material, or squirting, is also much more frequent when the tide is first coming in, as will be evident to anyone who will take the trouble to stand knee deep in the incoming tide in a clam bed and observe.

The mantle cavities of the four pelecypods were never free of mucus, and particles are at all times conveyed to and along the edges of the gills by mucus, but it is only at feeding times that the gills are covered by the sheets of mucus. While feeding is going on these sheets are being continuously secreted and move slowly toward the free edges of the gills.

It should be noted here that some mucus with its included particles may find its way into the mouth when the sheet of mucus is not present. I think this is sometimes due to testing the mucus for suitable food, for the secretion of the mucous plate often follows such testing. Abnormal ingestion of material often follows disturbance or mutilation. This is clearly shown by placing carmine or carborundum on the gills of a pelecypod which has been opened. Under such conditions the introduced material may be carried to the mouth and ingested. This never happens in the case of a pelecypod which is feeding normally in an aquarium as observed through a window, for the introduction of even small amounts of carmine or carborundum causes the pelecypod to cease feeding immediately. I consider carborundum particles the most undesirable of all materials to use in feeding experiments.

In connection with the above, I am of the opinion that the function of the osphradia as " water testing organs " is over-emphasized in the pelecypods. The region which seems to me to have the highest tactile and " olfactory " sense is the region where the incurrent opening is located. In *Mytilus, Pecten* and *Ostrea* it is the edge of the mantle; in *Schizothaerus* it is the finger-like papillae which partially close the entrance of the incurrent siphon and act as a coarse strainer. When a valve has been removed, part of the reception area for stimuli has been removed, and the nervous system of the pelecypod is rather badly upset, to say the least. What an animal does with the mucous threads from its gills at such a time had better be disregarded.

Discussion

It is unwise to speak of feeding in a pelecypod unless it is actually observed doing so. Pelecypods are very sensitive to stimulation, either mechanical or chemical (Hopkins, 1932*a*, 1932*b*), and sometimes will cease feeding at the least movement or change in food material. In general, I think it may be said that small or juvenile members of any species are less sensitive than the older and larger ones, for they adjust themselves more quickly to handling and begin feeding sooner after the window is placed in them. For this reason it is best to use as young specimens as one can conveniently. They are quite erratic for several days after being moved into the laboratory, and also after any major disturbance. However, when once fixed and left alone for a considerable length of time, which varies in each individual, they become much more uniform in their feeding activities, although apparently none of them ever feed continually. The rate of intake of water varies considerably, and this is usually or perhaps always accompanied by some contraction of the gills. Complete contraction of the gills shuts off the current of water entirely, just as a similar contraction does in the tunicate basket (MacGinitie, 1939). It is impossible for large particles to pass through the gills. Whether such small particles as bacteria pass through or not depends on whether or not the pelecypod is feeding.

It is well known that cilia often have a selective function, fine examples being the cilia of the pouch and funnel of *Stentor* (Schaeffer, 1910), and the egg and sperm collectors of *Urechis* (MacGinitie, 1935). Nevertheless, the separation of solid material from water currents is much more efficiently done by straining such water through mucus. It is not surprising, therefore, that mucus plays a much more important rôle in the feeding mechanisms of plankton and detritus feeders than it has been given credit for doing. Certainly it never should be said that a pelecypod is feeding just because it is pumping or maintaining a current through the mantle cavity.

In the light of the information presented here, it is interesting to read other papers concerned with the feeding of pelecypods and even certain gastropods (Orton, 1912). When the use of a mucous sheet for straining food material from the water is understood it will eliminate many points of discussion that have arisen. Although many papers concerned with the feeding of pelecypods have been written, only a few are listed in this paper, for the literature on this subject is rather voluminous. Therefore, the reader is referred to the following papers for complete bibliographies on the subject (Atkins, 1936–38; Galtsoff, 1928; Hopkins, 1936; Nelson, 1938; Orton, 1912; Yonge, 1936; and ZoBell and Feltham, 1938).

When it is understood that the food material of pelecypods in general is strained from the water as it passes through a sheet of mucus, and that feeding is being carried on only when such a sheet is present, it will clear up practically all points of uncertainty that one meets in reading about feeding methods and feeding experiments in the pelecypod mollusks.

Summary

1. Openings were made through the valve and mantle of four species of pelecypods. These were made in various positions in the valves of many individuals so that ultimately all outer portions of the feeding mechanisms could be observed. These openings were covered with pieces of cover-glass cemented in place so as to form windows through which the feeding activities could be watched.

2. The feeding activities were observed through a binocular without in any way disturbing the animals.

3. Evidence is given to show that when a pelecypod is feeding a sheet of mucus covers the gills, and it is this mucus which strains the food material from the water, the cilia affording mechanical means for its transportation.

4. While the pelecypod is feeding this mucus is constantly being secreted and is carried to the food grooves bordering the gills, along which it is transported to the mouth as strings of food-laden mucus.

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THE COLORATION AND COLOR CHANGES OF THE GULF-WEED CRAB, PLANES MINUTUS

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Many observers have noted the remarkable adaptations in color pattern of the gulf-weed fauna. Perhaps the most common and colorful crustacean associated with the gulf-weed is the little grapsoid crab, *Planes minutus* (L.). Its predominant color is brown of many shades from yellow to red, matching the weed to which it clings. The brown of many individuals is broken by conspicuous white patches of various shapes and sizes, some sufficiently large to cover the entire carapace. These white areas appear to be in imitation of the conspicuous calcareous tubes of the annelid worms attached to the gulf-weed. A color plate showing a few of the variations is given in Murray and Hjort's book (1912). Little experimental work has been done with this crab. Crozier (1918), who found some mahogany-colored *Planes* on a drifting tree of that shade cast ashore at Bermuda, was unable to detect any change in coloration after they had been kept for six days on the much lighter gulf-weed. The coloration and color changes of one of the other members of the gulf-weed fauna, the shrimp, *Latreutes fucorum,* have recently been described by Brown (1939).

In an attempt to discover whether the coloration of *Planes* is a fixed pattern or an active adaptation to background, crabs were kept on different backgrounds and observed microscopically as well as grossly. A white background was furnished by white china bowls. Other backgrounds were obtained by painting the outer surface of clear glass dishes. Ordinary commercial paints were used for this, the green, for example, being of a dark shade marketed as " window blind green." The crabs were exposed to these backgrounds for one day in full sunlight from dawn to dusk. At sundown the bowls were placed under a bright electric light until the crabs could be examined. Microscopic observations were made on the flattened surface of the fifth leg, a region easily viewed under low magnification (× 62). The results of these observations are summarized in Table I.

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There are three kinds of chromatophores in *Planes minutus:* white, black and yellow. Of these the most prominent are the white cells, which usually appear to be larger and more numerous than the black cells. The yellow cells are smaller than either the whites or blacks, and are hard to distinguish except in the contracted state, both because of their diminutive size and because of masking by the other chromatophores. In some individuals these cells appear to be almost orange in color, and when viewed in the contracted state through the darker areas of the leg they appear to be ruby. It is probable that the yellow pigment is astacin, a carotinoid commonly found in Crustacea. This pigment appears red when concentrated. It may be that the ruby appearance is caused in part by the fact that they are viewed through a region of the exoskeleton which is dark-brown in color.

TABLE I

Responses of chromatophores in Planes minutus to different backgrounds

	O	*A*	*B*	*C*	Number of Crabs Examined
White Background					
Black cells	0	1	4	27	32
White cells	8	18	4	2	32
Yellow cells	3	2	12	15	32
Black Background					
Black cells	27	0	3	0	30
White cells	0	3	9	18	30
Yellow cells	18	6	6	0	30
Red Background					
Black cells	24	5	6	0	35
White cells	3	12	8	12	35
Yellow cells	10	4	15	6	35
Blue Background					
Black cells	0	0	5	18	23
White cells	13	9	1	0	23
Yellow cells	2	0	4	17	23
Yellow Background					
Black cells	0	1	4	21	26
White cells	12	13	1	0	26
Yellow cells	1	0	11	14	26
Green Background					
Black cells	1	8	5	5	19
White cells	5	5	5	4	19
Yellow cells	4	0	11	4	19

Symbols: *O*—pigment fully dispersed; processes indistinguishable.
A—pigment partially dispersed; arborizations visible.
B—pigment partially concentrated; stellate.
C—pigment fully concentrated; punctate.

Symbols after Kleinholz (1937). See his paper for illustrations of the four phases of pigment distribution.

On most of the backgrounds tested each type of chromatophore reacted fairly consistently, its pigment tending to become either concentrated or dispersed. In general the black cells and the yellow cells reacted similarly, the only clear exception being when the animals were exposed to a red background. White cells and black cells reacted oppositely, the pigment of one becoming concentrated when that of the other became dispersed. The responses of the white cells to red and green backgrounds were not consistent, nor were those of the black cells to a green background. Perhaps examination of more animals or a longer exposure would have eliminated these apparent inconsistencies. However, it has been observed by others that there is in crustaceans some variability in chromatophoral response not only when different individuals are compared but also when different regions of the same animal are studied. The condition of the yellow pigment was frequently very difficult to determine, which introduced the likelihood of observational error.

Table II

Responses of Planes minutus compared with those of Portunus anceps and Portunus ordwayi

(Data on Portunus from Abramowitz (1935)).

Background	White Pigment Cells			Yellow Pigment Cells			Black Pigment Cells			Red Pigment Cells
	Planes minutus	*Portunus anceps*	*Portunus ordwayi*	*Planes minutus*	*Portunus anceps*	*Portunus ordwayi*	*Planes minutus*	*Portunus anceps*	*Portunus ordwayi*	*Portunus ordwayi*
White	D	D	D	C	C	C	C	I	C	C
Black	C	C	C	D	D	D	D	D	D	D
Blue	D	C	C	C	C	C	C	D	D	D
Red	C	C	C	C	D	D	D	D	D	D
Yellow	D	C	I	C	C	D	C	D	C	C
Green	—	D	C	C	C	C	—	D	D	I

Symbols: D—dispersed,
I—intermediate,
C—concentrated.

In spite of chromatophoral responses, *Planes* is unable to effect color adaptation rapidly, for animals kept all day on white or yellow backgrounds became but slightly lighter than those kept on black. Almost every individual from one background can be matched in coloration in a group from a contrasting background. It is interesting to note that Abramowitz (1935) observed similar behaviour in two cancroid crabs. One of these, *Portunus anceps,* has the same three pigments as *Planes,* while the other, *Portunus ordwayi,* has a fourth pigment, red. The red and black pigments were found to react similarly except perhaps to a green background, where the black became " dispersed " and the red " intermediate." Table II gives a comparison of the responses of *Planes* and the two species of *Portunus.* Since the responses of the cancroid crabs did not agree in all cases (one type of chromatophore apparently being concentrated in the one species and dispersed in the other for the same background), complete agreement between them and *Planes* is not found. However, if only those cases where the two species of *Portunus* are in agreement in their response are compared with *Planes,* there is lack of uniformity in only three instances: on a blue background for both the white and the red cells, and on a red background in the case of yellow cells. These differences are probably of no significance in view of the lack of consistent response which Abramowitz reported.

Direct comparison between the chromatophoral responses of *Planes* and *Latreutes* is difficult because the latter has red and blue pigments in addition to the white and yellow of *Planes,* and lacks black. The two species are alike in exhibiting a wide variety of colors and color patterns, but whereas *Planes* shows almost no alteration in appearance, *Latreutes* shows the effects of physiological color change almost immediately (Brown, personal communication).

The failure of *Planes* to effect a rapid color change in spite of its active chromatophoral responses may possibly be explained, at least in part, by a study of the moulted exoskeleton, which is a faintly colored replica of the intact skeleton. Each dark area or white spot on the intact animal is present in the exoskeleton, the intermediate areas being pale yellowish brown. Extra-chromatophoral pigment is found also in the hypodermal cells. Because of the distribution of this pigment, crabs whose chromatophores have reacted to a certain background do not become better color-adapted upon moulting. Until the diffuse pigment can be elaborated or destroyed, according to the prevailing condition of the chromatophore, it prevents the changed state of the chromatophores from becoming evident in the general appearance of the animal. This process apparently takes considerable time.

The pattern of the individual crab is probably genetic, as Brown has suggested for *Latreutes.* This is borne out by the observation made by Beebe (1928) that embryos of *Planes* have marked differences in pattern before hatching (p. 194). Yet it is clear from Crozier's report of the mahogany-colored individuals that *Planes* can in time become adapted to new backgrounds. The experiments reported above show that the chromatophores of *Planes* are responsive, but that extracellular pigment in the hypodermis and exoskeleton prevents the animal from effecting an immediate change in appearance.

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SOME EXPERIMENTS ON THE EFFECTS OF HYPOPHYSECTOMY AND PITUITARY IMPLANTATIONS ON THE MALE FUNDULUS HETEROCLITUS [1, 2]

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While sufficient work has been accomplished to show that the hypophysis of fish secretes a gonadotropic principle, the hormonal relationships involved in the piscine sexual cyle are not clearly understood (compare Matthews, 1940 for a summary). For *Fundulus* Matthews (1939) found that after hypophysectomy, the testes failed to continue gametogenesis during the breeding period. The injection of mammalian pituitary extracts had no decisive effect upon the gonads. Matthews (1940) found, however, that the implantation of *Fundulus* pituitaries into non-hypophysectomized immature *Fundulus* induced gametogenic activity, especially in the male. The present investigations are complementary to those of Matthews. Positive effects from pituitary implantations were secured in hypophysectomized adult male *Fundulus*.

Effects of Hypophysectomy

On July 3–6, 73 freshly captured, mature male *Fundulus* were hypophysectomized. The opercular approach was used for this very simple operation. Control fish were given blank operations. All fish were maintained under identical conditions in running sea water, the temperature of which varied between 11° and 19° C. The water for the most part was near or below 15° C. The fish were fed almost daily on chopped clams. This diet appeared adequate, since the operated fish deposited fat as do fish in nature during the summer.

Mortality in operated fish was about 30 per cent. No significant difference was found between the mortality of hypophysectomized fish and those which received a blank operation. Over a two-month experimental period, the loss of the hypophysis seems to have little to do with the viability of *Fundulus*.

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[1] Aided in part by a grant from the Penrose Fund of the American Philosophical Society; this grant administered in 1939–40 by T. H. Bissonnette.

[2] There is some doubt if this fish is strictly speaking, *Fundulus heteroclitus*. It may be a related heteroclitoid form.

At the time of hypophysectomy, the testes had passed their maximal development for the annual sexual cycle (Fig. 1). In nature, the maximal development occurring during the spring is followed by testicular regression. By late August, spermiogenetic transformations have almost ceased and the testes are practically devoid of sperm. As pointed out by Burger (1940), testicular regression does not occur as rapidly for fish kept in cool water (11°–17° C.) in the laboratory, as it does in the warmer water of the natural habitat. The degree of testicular regression attained by September 1, in fish which received blank operations, is shown in Fig. 2.

The effects on the testis after hypophysectomy were similar to those described by Matthews (1939) for other periods in the sexual cycle, viz., after complete testicular regression (October–December), and at the beginning of normal spermatogenesis (March–April).

The testes underwent a rapid reduction in size. The most obvious effect was a cessation of sperm formation. This cessation was not immediately a complete one. One month after hypophysectomy, however, only rare cysts of spermatids could be found. Two months after the operation, spermatids were absent in the six testes examined. At no time were spermatogonial multiplications suppressed. These divisions formed a well-defined cortical zone of spermatogonia. Cross-sections of testes from hypophysectomized fish are shown in Figs. 3 (August 2, one month after hypophysectomy), and 4 (September 1, two months after operation, cf. with control, Fig. 2). Thus it would appear that the loss of the hypophysis results in inhibition of spermatogenetic stages beyond those of spermatogonial division. Nevertheless,

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FIG. 1. Cross-section of a testis at time hypophysectomies were performed (July 3–6). This testis has passed its maximal sperm production. Black patches are sperm.

FIG. 2. Cross-section of a testis from a control fish, killed September 1; this fish has experienced a blank operation. Black patches are sperm. The cortical zone where spermatogenetic stages are visible, has become narrow (cf. Fig. 1).

FIG. 3. Cross-section of a testis from a hypophysectomized fish, killed August 2. The cortical zone contains almost nothing but spermatogonia. Spermiogenesis has practically ceased.

FIG. 4. Cross-section of a testis from a hypophysectomized fish, killed September 2. The cortical zone of spermatogonia has grown more narrow (cf. Fig. 3, one month earlier).

FIG. 5. Cross-section of a hypophysectomized fish implanted with twenty pituitaries, beginning August 17 and killed September 2. The spermatogenetic zone has deepened noticeably. Newly-formed sperm are visible. Compare with control, Fig. 4.

FIG. 6. Cross-section of a testis from a fish which received a blank operation; this fish was implanted with twenty pituitaries beginning August 17 and killed September 2. Compare with control, Fig. 2.

it does seem true that once spermatogenesis has been initiated, spermiogenesis can continue for some time, and to a limited degree, in the absence of the pituitary. The fact that this spermatogenesis is not maintained in any great volume even for as long as one month, indicates,

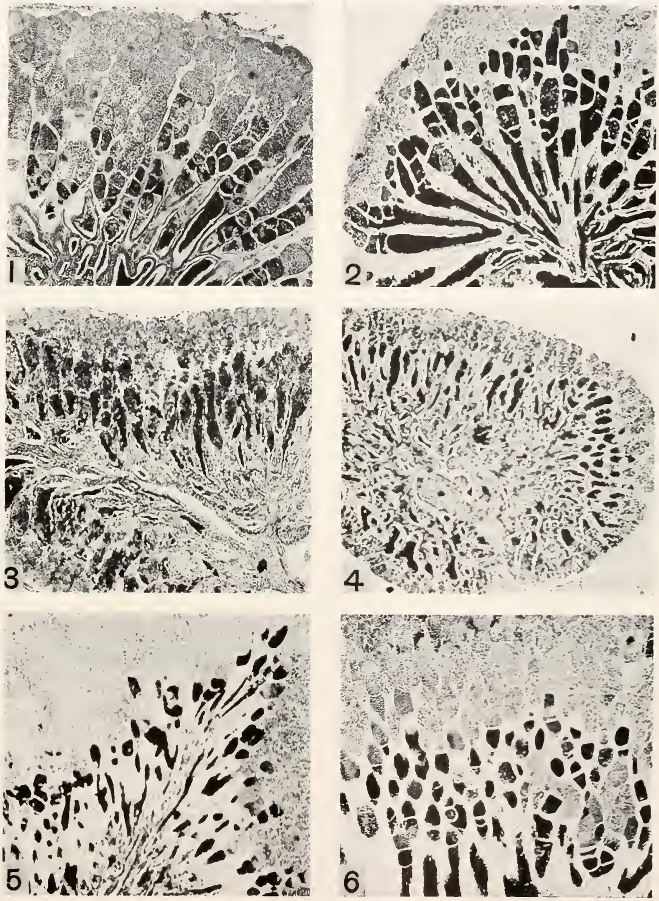

PLATE I

as Matthews (1939) has suggested, that the later stages of spermatogenesis are more sensitive to the absence of the pituitary than are the stages of spermatogonial division.

Effects of Pituitary Implantations

After the testes of the hypophysectomized fish had involuted sufficiently, both hypophysectomized fish and some operated controls were implanted with pituitaries from freshly captured, mature male *Fundulus*. The receptors selected were close to each other in size. The size of the donor fish was also roughly standardized. The implantations were made intraperitoneally. Ten hypophysectomized fish and three operated controls each received five fresh glands on each of the following days: August 17, 21, 26, and 29. These fish, which each received twenty glands, were sacrificed on September 2. Five hypophysectomized fish and two operated controls each received five fresh glands on each of the following days: August 21, 26, 29. These fish were killed on September 4. During this phase of the work, the water was almost constantly near 13° C.

The effects of the implantations were striking. Within five days after the first injections, the fish began to show pronounced sexual display antics. These peculiar swimming movements are common in fish during the breeding season and occur occasionally throughout the year in fish kept in aquaria. These movements were noticeably absent in the untreated hypophysectomized fish. By the tenth day after the first injection, the fish were in a frenzy of display.

Both series of implantations caused a recrudescence of the testes. After two weeks, the average weight of the testes of the fish which received twenty implants was slightly more than doubled, and the average volume was quadrupled, when compared with the average weight and volume of untreated hypophysectomized controls. The cortical zone of spermatogonia had deepened, while new transformations into spermatozoa were well established (Fig. 5). The hypophysectomized fish which each received fifteen glands likewise, formed new sperms. The weight and volume increases were the same as those for the fish which received twenty glands. The spermatogenetic stages were also the same. The non-hypophysectomized fish which were receptors of implants reacted as did the hypophysectomized receptors (Fig. 6). The control hypophysectomized fish (Fig. 4), and the control fish which experienced blank operations (Fig. 2) showed no testicular recrudescence during the experimental period.

These results clearly demonstrate that the pituitaries of adult male

Fundulus contain, and the testes of hypophysectomized and normal adult fish are responsive to, gonadotropic material. Matthews (1940) has shown that the immature testis of non-hypophysectomized *Fundulus* can be excited by Fundulus pituitaries implanted into the body cavity.

Discussion

The present study, together with that of Matthews (1940), permits an interpretation of the pituitary rhythm involved in the normal sexual cycle. During the period of sexual regression, pituitary secretion gradually declines. The pituitary does not abruptly cease to secrete, since active spermiogenesis in a gradually decreasing volume occurs during this regression. When the pituitary is removed, spermatogenesis is more quickly checked. In the late summer, pituitary secretion seems to be almost or entirely absent. Beginning in the fall and going through the winter, spermatogonial multiplications take place. The divisions can occur in the absence of the pituitary, but in the hypophysectomized fish there is no progressive increase in the number of these spermatogonia. Hence, the piling up in the testis of spermatogonia must be supported by hypophyseal secretion. The amount of this secretion is low, since very few spermatozoa are formed during the fall and winter. The spring spermatogenesis is accompanied by the highest phase of secretion. The gonadotropic material of the *Fundulus* pituitary is thus responsible for two phases of spermatogenesis: (1) the great volume of spermatogonial proliferation, and (2) maturation phenomena. It has been shown by Burger (1939) and Matthews (1939*a*) that the progressive phases at least of the sexual cycle of the male are influenced by the temperature of the water.

Summary

Adult male *Fundulus,* hypophysectomized shortly after maximal testicular development, show an inhibition of spermatogenesis for stages beyond those of spermatogonial multiplication. Spermatogonial divisions do not become numerous. The inhibition of the later stages is not immediately affected, since a few cysts continue to form sperm for as long as one month after hypophysectomy. These results confirm those of Matthews.

Both hypophysectomized adult male *Fundulus* and fish which received blank operations were maintained until sexual regression was well established. Implantations of twenty or fifteen pituitaries from normal male *Fundulus* caused within two weeks a recrudescence of the testes. Non-implanted controls showed none of this activity. It is concluded

that the pituitary of the adult male *Fundulus* contains gonadotropic material and that the testes of adult *Fundulus,* hypophysectomized or not, are responsive to this material.

The normal relation of the pituitary to the annual sexual cycle is discussed.

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MELANOSIS IN THE COMMON COD, GADUS CALLARIAS L., ASSOCIATED WITH TREMATODE INFECTION[1]

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A codfish displaying an unusual degree of melanosis is described in this paper. The fish was caught one mile north of Race Point, Provincetown, in March, 1940, by Mr. J. W. Lowes, who sent it to the Museum of Comparative Zoölogy. Mr. William C. Schroeder asked the writer to make a histological study of the tegumentary system of the specimen in a search for a possible clue to the cause of its melanosis.

METHOD

Samples of skin of one-half to one centimeter square were taken from different parts of the head, trunk and fins. The regions employed are indicated by letters and numbers shown in Fig. 1. Each sample was dehydrated, cleared and mounted in balsam. With a calibrated ocular micrometer ruled into squares, 3 to 5 separate square millimeters from each piece of skin were measured under a binocular microscope, using reflected light, and the number of pigment cells per square millimeter determined and recorded from each region.

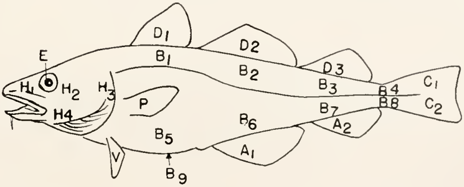

FIG. 1. Diagram of left side of cod showing regions from which skin samples are removed for comparative study. *A*, anal fin; *B*, body or trunk; *C*, caudal fin; *D*, dorsal fin; *E*, eye; *H*, head; *P*, pectoral fin; and *V*, ventral fin.

Small pieces of skin from the first dorsal fin (region *D 1*), the trunk, directly ventral to the first dorsal fin (region *B 1*), and the cornea (region *E*) were sectioned and stained with Heidenhain's "azan" stain which gives a blue color to the connective tissue, reddish yellow to the muscles, carmine to the cyst wall formed by the host tissue, blue to the cyst wall secreted by parasites and blue and carmine to the parasites themselves.

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[1] Contribution No. 253 of Woods Hole Oceanographic Institution.

[2] China Foundation Research Fellow.

For comparison a normal cod was treated in the same way.

The trunk muscles, gills, oesophagus, heart and peritoneum were examined for parasites. As the fish had been eviscerated, only remnants of the cardiac portion of the stomach and liver were examined. The cysts were isolated, stained, and mounted and some of them sectioned and stained.

OBSERVATIONS

Superficial examination of the whole fish and microscopic study of sections of its skin show that general cutaneous melanosis in this fish is associated with parasitic infection which attacks the whole tegumentary system and the gill filaments. No parasitic cysts were found in the somatic muscles, the peritoneum, the heart, the oesophagus, the remnants of the liver and the cardiac end of the stomach which happened to be left in the fish after its evisceration. These uninfected regions exhibit no melanosis when compared with the corresponding regions in the normal cod.

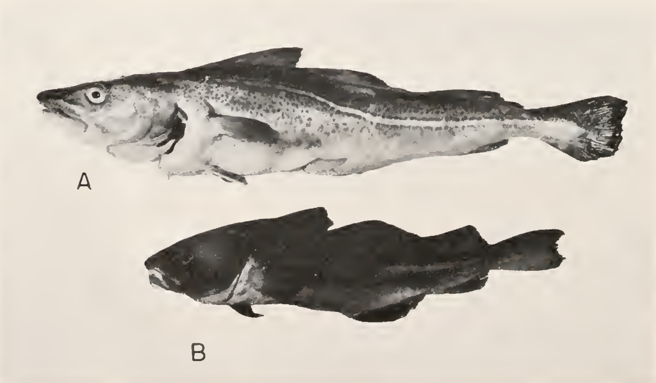

FIG. 2. *A*. Normal cod. *B*. Dark cod described in this paper.

In general appearance this fish is strikingly different from an ordinary cod in the presence of so many melanophores in the corneae and in the skin over the fins and the dorsal half of the body that these parts are actually black. The contrast in color between this and normal cod is shown in *A* and *B* of Fig. 2, which are printed from one photographic negative and hence are of identical exposure. Instead of being smooth and shiny, the skin is warty and rough. The tiny excrescences which produce the roughness are covered with more melanophores than the surrounding tissues. Parasitic cysts appear as white specks among the melanophores. Inside the cysts different stages of the metacercaria of a heterophyid trematode are seen.

The cysts and melanophores are so abundant in the cornea that the fish is blind and the eye scarcely distinguishable from the rest of the head. The melanophores on the body above the lateral line and on the dorsal and caudal fins are so numerous that they form a continuous sheet, making it impossible to ascertain their number per unit area of skin. In the less densely pigmented regions the dark cod has, on the whole, about six times as many melanophores per square millimeter on the head and six to nine times as many on the paired and anal fins as has a normal cod. Table I shows the number of pigment cells per square mm. for each of the 23 corresponding samples of skin from the dark and normal cod. The last two columns show that the melanophores of the dark cod are smaller than those of the normal fish. In the dark fish the melanophores are more uniform in size. In Fig. 3, *A* and *B*, two equal pieces of skin from the pectoral fin of a normal cod and this dark cod are compared. The normal cod has only one-sixth as many pigment cells as the dark individual.

Parasitic cysts are present in the tegumentary system from the tip of the snout to the surface of the caudal fin, including both corneae. When examined under a dissection microscope, they appear as small white dots among thick masses of melanophores—the tips of the cysts being free from pigment cells. From Fig. 3, *C*, it will be seen that the rugose appearance of the skin is produced by a mass of parasitic cysts under the epidermis which is thrown into folds. These cysts occur both above and below each scale, which, when pulled off from the body, always has a mass of cysts firmly attached to its two surfaces. The connective tissues are hypertrophied so that the skin is more than three times as thick as the normal skin from a corresponding part of the body (Fig. 3, *D*). The cornea is also infested. In Fig. 3, *F*, which is a photomicrograph of a 4 μ thick section of a piece of cornea, 10 trematode cysts can be seen from a field 1.86 mm. long and 0.66 mm. wide. This cornea is more than twice as thick as a normal one and has melanophores throughout its whole thickness.

TABLE I

Comparison of melanophores between dark and normal cod

Body region	Number of melanophores		Ratio of melanophores: $\frac{\text{Dark}}{\text{Normal}}$	Size of melanophores in mm.	
	Normal	Dark		Normal	Dark
Head:					
H 1	25	121	5	0.15–0.2	0.1
H 2	10	72	7	0.3	0.1–0.2
H 3	12	72	6	0.3	0.15
H 4	9	63	7	0.3	0.25
Trunk:					
B 1	46	Too numerous		0.2	0.05–0.1
B 2	51			0.2	0.05–0.1
B 3	55			0.2	0.05–0.1
B 4	82			0.1	0.1–0.15
B 5	7			0.3–0.4	0.1–0.15
B 6	7			0.3	0.15–0.2
B 7	17			0.2	0.2–0.25
B 8	14			0.4	0.15–0.2
B 9	1	19	19	Contracted	0.2–0.3
Dorsal fin:					
D 1	48	Too numerous		0.15–0.2	0.1
D 2	39			0.2	0.1
D 3	55			0.15–0.2	0.1–0.2
Anal fin:					
A 1	5	40	8	Contracted	0.2–0.3
A 2	5	51	10	0.2–0.4	0.2
Caudal fin:					
C 1	87	Too numerous		0.1	0.1
C 2	45			0.15–0.2	0.1
Pectoral fin:					
P 1	5	46	9	0.4	0.1–0.15
Ventral fin:					
V 1	6	37	6	0.2–0.25	0.2
Eye:					
E 1	0	66		0	0.2

The parasitic cysts are thick-walled, ovoid in outline and white to the naked eye. The majority of the cysts measure 0.33–0.38 mm. along one principal diameter and 0.24–0.28 mm. along the other. The cap-

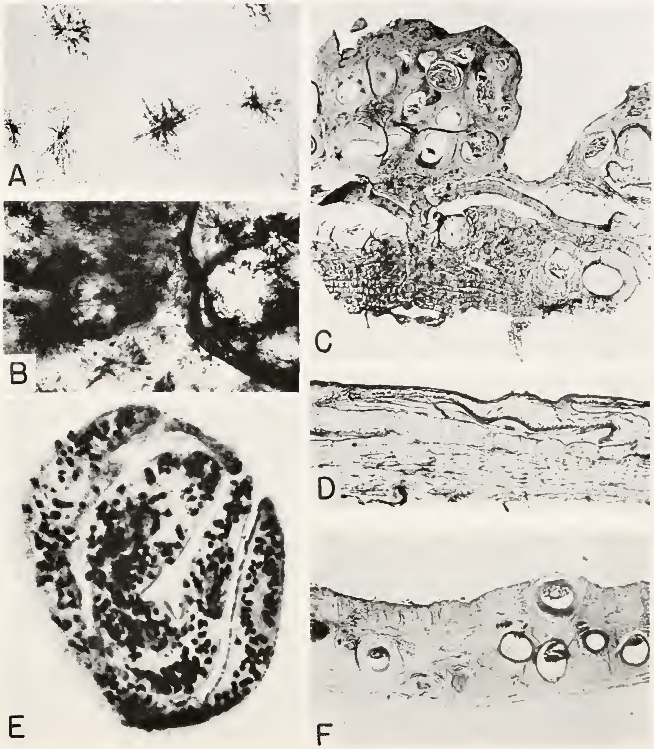

Fig. 3. Photomicrographs of preparations from cod skin.

A. Skin from the pectoral fin (same region as in *B*) of a normal cod.

B. Skin from pectoral region (*P* 1) unstained, showing part of the outline of two cysts and melanophores.

C. Section of a piece of melanotic cod skin from region *B* 1 (below the first dorsal and above the lateral line), showing the wavy epidermis and clusters of cysts. Section of one scale is shown in this figure. Three cysts are seen under the scale, while above it there is a large number of cysts.

D. Skin of normal cod from the same region and under the same magnification as in *C* above, showing one scale in section and part of two others and the smoother epidermis and less connective tissue.

E. Section of a metacercaria.

F. Section of the cornea showing cysts and pigmentation.

sules secreted by the parasites to enclose themselves measure 0.21 mm. by 0.14 mm. in the two principal diameters. The cyst walls are very resistant to mechanical injuries and are transparent in unstained skin cleared in xylene and mounted *in toto* in balsam. The cyst wall secreted by the host stains bright carmine with Heidenhain's "azan" stain and is laid down in concentric layers. It measures 0.028–0.07 mm. in thickness. The cyst wall secreted by the parasite, on the other hand, is only about 0.007 mm. thick and stains blue. In the gill filaments many smaller cysts are seen. This difference in size is due to the thinness of the host wall, for the smaller cysts contain parasites which, when measured along the parasite wall, are of the same size as those enclosed in the larger cysts of the gill filaments or the skin of the trunk. The host wall about the parasite increases in thickness, with more concentric layers, as the external size of the cyst increases. There is comparatively very little pigmentation about the cysts in the gill. All the melanophores present in the gill are arranged about the cysts with thick host walls. As the host walls are laid down about the parasites in concentric layers centrifugally and as the melanophores are associated with the more peripheral layers, it is probable that these pigment cells appear some time after the infection occurs.

The cyst contains the coiled body of the metacercaria of a trematode whose suckers can be distinguished through the transparent cyst walls. In stained sections (Fig. 3, *E*) the suckers and the spines on the posterior part of the body wall of the parasite can be seen easily. But as the metacercariae are still young, it is not possible to work out the structures of the reproductive system of our material.

From the absence of the parasitic cysts inside the body of the fish except in the tegumentary system and the gills, it may be inferred that the trematode larvae infected the cod by boring from the outside. The presence of very thin-walled parasitic cysts in the gill filaments indicates that these were the latest site of infection.

Compared with other infected fishes, this cod shows an extraordinarily heavy infection. Not only is the whole tegumentary system completely infested with parasites, but the parasitic cysts are gathered in groups several layers thick under the epidermis. According to Dr. Stunkard (verbal communication), a cunner kept in a laboratory aquarium for six weeks with 50 infected snails giving off thousands of trematode larvae does not get nearly so heavily infected. It is the more surprising when we consider that although in the ocean the cod was able to move about, it nevertheless contracted such an enormous number of parasites.

Discussion

Many cases of melanosis associated with parasitism have been observed among freshwater fishes. There are also a few records of melanosis in marine fishes parasitized by trematode larvae. In 1884 Ryder reported his observations on the darkening of the skin in parasitized cunners from Woods Hole and Cape Breton, N. S. He thought that these cysts were formed by the cercariae of some trematode and that the pigment cells about the site of infection were either formed *de novo* or gathered there by migration. Linton (1900) observed parasitic cysts on the skin of cunners in 1889, and in 1901 he reported similar infections on tautog, winter flounder, tom cod and eel and " less so on others." In 1915 he recognized the similarity between these encysted forms and the trematode *Tocotrema lingua* (Creplin). The presence of this species on the gills of sea raven and on the skin of cunners from Passamaquoddy Bay was reported by Cooper (1915). Stunkard worked out experimentally (1930) the life history of this trematode and identified it as *Cryptocotyle lingua* (Creplin), belonging to the family Heterophyidae—*Tocotrema* being long suppressed as a synonym of *Cryptocotyle*. Smith (1935) described a hyperplastic epidermal disease in two winter flounders associated with a trematode infection which was probably due to *Cryptocotyle lingua*. All these observations were probably concerned with the same species. A second species was reported by Gamble and Drew (1911) from Plymouth. A whiting infected by trematode larvae showed abnormal pigmentation in the form of black specks scattered over its pigmented areas and over the conjunctiva. They suggested that the trematode was a species of *Holostomum*, probably *H. cuticola*. No melanosis due to parasitism in the cod has been reported. In the cod described here the heaviness of infection and the intensive reaction of the host in the hypertrophy of the skin and the development of excessive melanophores are very remarkable.

Smith's experiments (1931, 1932) on the evoking of melanophores through mechanical injury and the eruption of corial melanophores and general cutaneous melanosis strongly suggest that these reactions are related to repair and defense of the tissues. The general eruption of melanophores in this cod, whose tegumentary system was completely infected by trematode metacercariae, is probably a defensive reaction against the parasite. There are different views on the question whether the melanophores in a parasitized fish migrate to the site of infection or are formed *de novo*. In this particular cod the number of melanophores is so much in excess of that found in an ordinary cod and they so completely cover the whole body that migration of melanophores cannot account for them. They must have developed anew.

The development of the metacercariae is not advanced enough to allow an exact identification of the parasite. However, the mode of reaction of the host and the structures of the parasite, as far as they can be determined, appear very similar to what Linton described for *Cryptocotyle lingua*, which is found to infect a variety of fishes such as cunner, tautog, tom cod, eel, sea raven, winter flounder, etc. They suggest that this cod is parasitized by a species related to *Cryptocotyle*.

SUMMARY

A very melanotic codfish is described which proved to be heavily infested with metacercariae of a heterophyid trematode. The number of parasites and the intensive reaction of the host in the development of melanophores and hypertrophy of the dermis are greater than any recorded for parasitized fish. The parasite may be a form related to *Cryptocotyle*.

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REPAYMENT OF THE ANAEROBIC OXYGEN DEBT IN GRASSHOPPER SKELETAL MUSCLE

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It has been shown (Gilmour, 1940) that in the roach *Cryptocercus punctulatus* Scudder, the "oxygen debt" incurred during a period of anaerobiosis is repaid threefold. Since such a large excess oxygen consumption during recovery had not previously been demonstrated in animal tissues, it seemed that further work on the phenomenon of oxygen debt repayment in insects was warranted.

In order to simplify the problem, it was decided to limit this investigation, as far as was possible, to one tissue. To this end, the hind femora of grasshoppers were employed as material. The femur of the jumping leg of the grasshopper consists almost entirely of skeletal muscle; the amount of epidermal and other tissue present would account for only a very small fraction of the total respiration.

MATERIAL AND METHODS

Two species of grasshopper were used: (1) *Melanoplus femur-rubrum* (De Geer). Adults of this species were collected in the field during the fall of 1939 and kept in the laboratory until used. (2) *Melanoplus differentialis* (Thomas). A number of adults were raised from eggs supplied by Dr. J. H. Bodine from the stocks at Iowa State University.

The legs were removed from the bodies by cutting through the trochanter. The tibiae were cut off close to the proximal end.

Oxygen uptake was studied in a differential volumeter designed especially for following oxygen debt repayment, the "apparatus B" previously employed by Rotta and Stannard (1939). The electrical conductivity method of Fenn (1928) was used in following carbon dioxide production. Oxygen uptake and carbon dioxide production of the resting leg were first followed in air, after which the vessels were filled with nitrogen, by running through the pure gas for 20 minutes, and the carbon dioxide production during anaerobiosis was determined; finally air or oxygen was re-admitted and the recovery respiration followed until a steady rate of oxygen uptake had been reestablished.

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It was not possible to use the respirometer designed for conductivity determination for following both oxygen uptake and carbon dioxide production during the recovery period, as the oxygen uptake readings were disturbed by solubility effects. The anaerobic period was ended by passing pure oxygen through the vessel for one minute, insufficient time for equilibrium to be established between the gas mixture and the relatively large volume of barium hydroxide required by the method. Oxygen uptake was followed, however, at the beginning and end of each run with the conductivity apparatus, in order to establish the respiratory quotient.

TABLE I

Repayment of oxygen debt in isolated hind femora of *Melanoplus femur-rubrum*. The duration of anaerobiosis sometimes varied slightly from that shown in column 2. Such variations have been considered in calculating the amount of oxygen missed.

Experiment	Time in N_2	Initial O_2 uptake	O_2 missed	Final O_2 uptake	Excess O_2 consumed	$\frac{\text{Excess } O_2 \text{ consumed}}{O_2 \text{ missed}} \times 100$
	minutes	*mm.³/gm./hr.*	*mm.³/gm.*	*mm.³/gm./hr.*	*mm.³/gm.*	
G3	30	218	110	180	193	175
G5	30	180	90	173	160	180
G6	30	186	99	179	160	160
G9	30	269	135	277	250	185
G12	30	191	96	180	262	275
G4	60	158	158	162	173	110
G8	60	140	140	166	196	140
G10	60	201	201	162	550	275
Average		193		185		190

The temperature of the experiments was 23° C.

That an adequate supply of oxygen to the interior of the excised legs was maintained by diffusion from air was demonstrated by the fact that filling the vessels with oxygen caused no increase in oxygen consumption above that measured in air. The legs survived without any apparent disturbance in respiratory metabolism throughout the course of the experiments (5 to 9 hours). The oxygen uptake usually remained constant over long periods of time, although the final steady rate was often slightly lower than the initial.

Experimental Results

M. femur-rubrum

Either two or three legs were used in each experiment. The average oxygen uptake was 180 cu.mm. per gram live weight per hour (26 determinations ranging between 140 and 277 cu.mm. per gram per hour). The results of the oxygen debt experiments are shown in Table I. "Oxygen missed," in this table, means the amount of oxygen the tissue would have consumed in air, during the time it was in nitrogen. The last column represents the percentage repayment of the oxygen debt.

Table II

Repayment of oxygen debt and retention of carbon dioxide in isolated hind femora of Melanoplus differentialis.

Experiment	Initial O_2 uptake	RQ	Final O_2 uptake	RQ	Percentage repayment of O_2 debt	CO_2 produced in N_2	Expected excess CO_2 production	Measured excess CO_2 production	CO_2 retained
	mm.³/gm./hr.		*mm.³/gm./hr.*			*mm.³/gm.*	*mm.³/gm.*	*mm.³/gm.*	*mm.³/gm.*
M1	178	—	164	—	120	—	—	—	—
M2	180	0.93	180	0.80	—	136	225	212	13
M5	132	—	134	—	175	—	—	—	—
M6	142	0.79	132	0.85	—	126	178	174	4
M7	198	—	186	—	115	—	—	—	—
M8	212	0.88	216	0.79	—	154	265	20	245
M10	205	0.94	183	0.85	—	116	256	88	168
M13	207	—	195	—	160	—	—	—	—
M14	243	0.67	234	0.67	—	100	304	166	138
M15	253	—	212	—	80	—	—	—	—
M16	277	0.72	237	0.73	—	87	346	194	152
M17	202	—	201	—	110	—	—	—	—
M18	187	0.98	207	0.89	—	123	234	176	58
Average	201	0.84	191	0.80	125	120			111

There is no significant difference between the value for this obtained from the half-hour experiments and that from the one-hour experiments.

M. differentialis

One leg was used in each experiment. The procedure was to use the femur of one side of the grasshopper in the oxygen debt apparatus, and that of the other side in the respirometer designed for conductivity determination.

The average oxygen uptake was 197 cu.mm. per gram per hour (29 determinations ranging between 132 and 277 cu.mm. per gram per hour). Table II shows the results for both oxygen uptake and carbon dioxide production. The percentage repayment of the oxygen debt is calculated in the same manner as in Table I. The duration of anaerobiosis was one hour in all experiments. The " expected excess carbon dioxide production " is the amount of carbon dioxide which would have been given off, over and above that produced as the result of basal metabolism, if the recovery process had had a respiratory quotient of 1.0, and there had been no retention. The carbon dioxide retained in the tissues during recovery is the difference between this figure and the measured excess carbon dioxide production. The figures obtained in this way show an extremely wide range of variation, but have an average which is approximately equal to the average amount of carbon dioxide produced during anaerobiosis. The variation must be due largely to the fact that oxygen uptake and carbon dioxide production during recovery were not determined on the same tissue. In determining the expected excess carbon dioxide production it was supposed that the repayment of oxygen debt had the average value (125 per cent) in each case; that is, that the figure for excess oxygen consumed (and hence for carbon dioxide produced) was 125 per cent of the original oxygen uptake per hour. It might be supposed that a more accurate method would be to use, in each of the carbon dioxide experiments, the figure for oxygen debt repayment obtained from the opposite leg of the same grasshopper. When this is done, however, the variation is as great, while the average is practically unchanged (106 cu.mm. per gram). It appears, then, that it is impossible to predict the actual percentage repayment of oxygen debt of any leg, even from an experiment run on another leg of the same grasshopper. The average is thus the only figure for carbon dioxide retention that need be considered.

The respiratory quotient is somewhat low for muscle, but at the time at which experiments M14 and M16 were run the insects were rather inadequately fed, and the low respiratory quotients in these experiments are probably the result of the utilization of reserve foodstuffs.

Discussion

Since the chemical constituents of insect muscle are quite similar to those of vertebrate muscle, it is not unreasonable to expect the anaerobic processes of the two groups to be at least qualitatively similar. The fact that in *M. differentialis* the amount of carbon dioxide produced during anaerobiosis is equal to the amount retained during recovery suggests that the amount produced in anaerobiosis is the result simply of the buffering of acid by bicarbonate, and supports the conclusion that lactic acid is the only important end-product of anaerobiosis. In frog muscle 70 per cent of the debt incurred during anaerobiosis is repaid (Rotta and Stannard, 1939). In the insects used in this study more than 100 per cent is repaid. The problem of the removal of lactic acid thus seems to be a more expensive one in grasshopper muscle than in frog muscle, particularly in the case of *M. femur-rubrum,* which uses more oxygen in the recovery process than does *M. differentialis.* It has already been suggested that in *Cryptocercus* (*loc. cit.*) the threefold repayment of the oxygen debt may have been due to the burning off of a large proportion of the lactic acid supposed to have been produced by anaerobiosis. The fact that in isolated muscle tissue, investigated at normal temperature (as *Cryptocercus* was not), a repayment of the oxygen debt in excess of 100 per cent can be demonstrated lends support to this conclusion.

SUMMARY

The average oxygen consumption of isolated hind femora of *Melanoplus femur-rubrum* was 180 cu.mm. per gram per hour; that of femora of *M. differentialis* was 197 cu. mm. per gram per hour. The average respiratory quotient of the latter was 0.82.

In *M. femur-rubrum* 190 per cent of the oxygen debt incurred during anaerobiosis was repaid during recovery. In *M. differentialis* 125 per cent of the debt was repaid, and the carbon dioxide retained in the tissues during the recovery period was equal to the carbon dioxide produced during anaerobiosis.

The end-products of anaerobiosis in grasshoppers are probably similar to those in vertebrates, but their removal seems to involve a greater expenditure of energy.

My thanks are due to Dr. W. O. Fenn for making available the facilities of his laboratory, and for his interest in the course of this work.

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GROWTH STUDIES ON CILIATES

VII. Comparative Growth Characteristics of Four Species of Sterile Ciliates

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During the past year experiments have been conducted on four species of holotrichous ciliates in pure culture in order to establish their nutritional requirements and some of their characteristics of growth. It is now possible to report the results of these experiments and to attempt an analysis of some of the factors of growth, both favorable and unfavorable.

In the ever widening field of protozoan physiology the quest is going on for more species which can be used for precise experiments. These species should be able to grow and reproduce in the absence of other microorganisms if complete control is to be obtained. Up to the present time it seems likely that the only genus of ciliate which has remained in successful pure culture is *Tetrahymena* (Furgason, 1940). Various names have been applied to pure-culture ciliates by different authors but, as Furgason has succeeded in showing, they were probably dealing with strains of *Tetrahymena geleii.* In a previous paper of this series (Kidder, Lilly and Claff, 1940) a description was given of a saprozoic ciliate which was referred to the genus *Glaucoma.* This organism (*G. vorax*) was described before access was had to Furgason's excellent work. I am now of the opinion that our ciliate belongs to the genus *Tetrahymena* and therefore it will be referred to in the future as *Tetrahymena vorax.*

Paramecium bursaria was cultured bacteria-free by Loefer (1936) but these cultures were subsequently lost. Because of the inclusions of *Chlorella* in this species the status of "pure culture" is questionable.

The four species to be dealt with in the following report are *Tetrahymena geleii* (strain W), *T. vorax, Glaucoma scintillans* and *Colpidium campylum.* All of these organisms were sterilized and established in pure culture in this laboratory and remain available to other investigators who may be interested in them for experimental purposes.

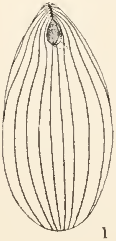
MATERIAL AND METHODS

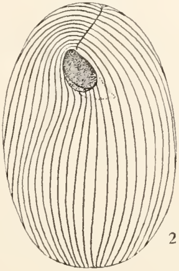
Isolation and Sterilization

Tetrahymena geleii (strain W) was isolated from Mill Pond in Woods Hole, Massachusetts in July, 1939. It was sterilized in the migration-dilution apparatus described by Claff (1940) and established in pure culture.

Tetrahymena vorax is the strain previously described from this laboratory (Kidder, Lilly and Claff, 1940).

Glaucoma scintillans (strain A) was isolated from Mill Pond in July, 1939. It was sterilized in the migration-dilution apparatus of Claff and established in pure culture. Strain B was isolated from a freshwater stream near Providence, Rhode Island in May, 1940. It was sterilized and established in the same manner as strain A. Strain B will be discussed only in reference to adaptation to sterile conditions as it was not used in any of the other comparative studies.





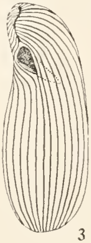


FIG. 1. *Tetrahymena geleii* (strain W). × 800. Total number of ciliary meridians = 17 — 19.

FIG. 2. *Glaucoma scintillans.* × 800. Total number of ciliary meridians = 35 — 40.

FIG. 3. *Colpidium campylum.* × 800. Total number of ciliary meridians = 27 — 30.

Colpidium campylum was isolated from a freshwater stream near Providence, Rhode Island in September, 1939. It was sterilized by migration across a fluid-filled Petri dish (details of this method given elsewhere, Kidder, 1940) and established in pure culture.

Description of Species

Because of the confusion which has resulted from lack of adequate description of experimental material, three figures are presented which show the diagnostic characteristics of those strains which were used and which have not been figured previously. These figures were prepared from opal blue treated material and indicate the distribution of the ciliary lines and the position of the mouth. Figure 1 is of *Tetrahymena geleii* (strain W) and corresponds almost exactly to the figures given by Furgason (1940) for this species. Figure 2 represents *Glaucoma scintillans* and Fig. 3 *Colpidium campylum*. No figure is given of *Tetrahymena vorax* as descriptions have been previously presented (Kidder, Lilly and Claff, 1940).

Studies of *Glaucoma scintillans* and *Colpidium campylum* have been made using the silver technique of Klein (1926) and the relief method of Bresslau (1922). Comparisons with slides prepared from different strains which were used in a previous study (Kidder and Diller, 1934) show that the present organisms are the same species as those reported at that time.

Conditions of Experiments

All qualitative studies were made from cultures grown in the specially designed Pyrex flasks described in detail elsewhere (Kidder, 1941). Organisms were counted by the direct method after appropriate dilutions. Qualitative observations were carried out on material grown in Pyrex test tubes.

Incubation of all experimental cultures was at 27° C. ± 0.2°.

Except in the experiments designed to test the effects of the age of the inoculum, all cultures were started from logarithmic phase ciliates. For uniformity the following ages of inocula were always used: *Tetrahymena geleii* (strain W)—18 hours; *T. vorax*—24 hours; *Glaucoma scintillans* and *Colpidium campylum*—48 hours.

Sterility tests on solid and in liquid media, incubated at room temperature and at 37° C., were carried out according to the methods outlined in previous studies of this series (Kidder and Stuart, 1939; Kidder, Lilly and Claff, 1940; Dewey and Kidder, 1940; Kidder, 1941) and, unless otherwise stated, all cultures were bacteria-free.

Method of Evaluation of Data

Attention should be called to an important point regarding the presentation and evaluation of data. The method often employed (Hall and Elliott, 1935; Hall, 1939; Hall and Schoenborn, 1939; etc.) of comparing the final concentration of cells (X) to the initial concentration (Xo) and expressing the result as the ratio X/Xo may lead to erroneous conclusions. The time selected for the final concentration count is arbitrary and may represent a point on the growth curve beyond the cessation of logarithmic growth. No information is obtained regarding the activity of the cultures during the earlier phases of growth. The same criticisms apply to the method developed by Elliott (1939) where total protoplasmic volumes are compared, unless estimations are made in the early stages of the growth of the cultures. Therefore it seems not only desirable but necessary to follow the growth of cultures by taking numerous samples at regular intervals. The culture flasks used in these experiments were designed for such a procedure (Kidder, 1941).

Experimental Results

Physical Condition of Medium

Tetrahymena geleii (strain W) and *T. vorax* are both able to utilize dissolved proteins. This fact was immediately apparent upon the initial sterilization. They began rapid reproduction when placed in any of the standard peptone media or in Difco yeast extract. The addition of particles to such media did not increase the growth rate or the yield. These ciliates correspond to the other strains of *Tetrahymena,* therefore, in their ability to grow and reproduce in dissolved materials. Evidence is still lacking regarding Lwoff's (1932) contention that saprozoic ciliates are able to take in polypeptides through the pellicle. We still do not know whether extracellular enzymes are released which might hydrolyse proteins. If it can be shown that no such proteolytic enzymes are released into the medium, then it seems fairly certain that nutritive materials, even in the dissolved state, enter food vacuoles by way of the cytostome. This conclusion would be justified when it is noted that at least five strains of *Tetrahymena* (tested by V. C. Dewey in this laboratory) have been found to exhibit perfectly normal growth characteristics in dissolved casein. It seems highly improbable that whole protein molecules could be absorbed through the pellicle.

Both *Glaucoma scintillans* and *Colpidium campylum* are dependent upon particles of nutritive materials. This fact was noted by E. and M. Chatton (1923) for *G. scintillans* when they were able to obtain growth on dead *B. coli* but not on dissolved proteins. Hetherington (1933) reports the establishment of *G. scintillans* in yeast autolysate but the ciliates failed to reproduce beyond a few divisions and the cultures were presumably discarded.

When *Glaucoma scintillans* (strain A) was first sterilized single ciliates were placed in 2 per cent proteose peptone broth. After many days only a few divisions had occurred and it was apparent that the medium was inadequate. Difco yeast extract (1 per cent) and liquid yeast autolysate (10 per cent) were no better. Those ciliates placed in particulate Yeast-Harris (Kidder, Lilly and Claff, 1940; Kidder, 1940), however, reproduced quite rapidly while those placed in a mixture of Yeast-Harris and proteose peptone yielded thriving cultures (Fig. 4). Yeast-Harris or the mixture with proteose peptone which had been rendered particle-free by filtration gave no growth. It was later found that strain B and *Colpidium campylum* likewise require particles in the medium and it was in this way that pure cultures of these strains were established.

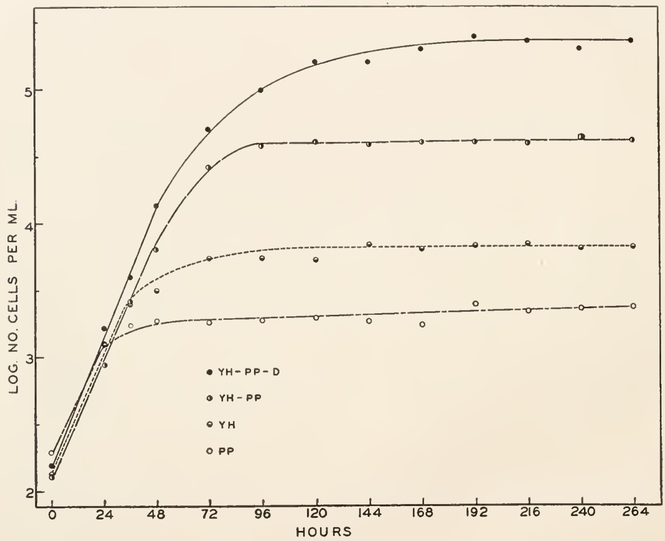


FIG. 4. *Glaucoma scintillans.* Growth curves constructed from the average data of 5 separate experiments. YH = 1 per cent Yeast-Harris; PP = 2 per cent proteose peptone; YH — PP = 1 per cent Yeast-Harris plus 2 per cent proteose peptone; YH — PP — D = 1 per cent Yeast-Harris plus 2 per cent proteose peptone plus 0.5 per cent dextrose.

Glaucoma and *Colpidium* appear to possess feeding mechanisms which are stimulated to ingestion only by solid particles. The slight amount of growth obtained in proteose peptone broth can be accounted for by the few particles which are invariably present after sterilization. When these particles are used up reproduction ceases.

After considerable experimentation the following basic medium was adopted as the best for general use in dealing with *Glaucoma* and *Colpidium*—

Brewers Yeast-Harris	10 grams
Pyrex distilled water	1 liter

This is brought to a boil and filtered, first through cotton and then through Schleicher and Schüll No. 595 filter paper. This does not remove the finer particles of the broken yeast cells and the resulting solution is slightly turbid. To this liter of 1 per cent Yeast-Harris is added 20 grams of Difco proteose peptone and the whole sterilized in the autoclave at 15 pounds pressure for 20 minutes. This forms the base for the other experimental materials or may be used without additions. The concentrations given appear to be near optimum for these two species of ciliates as both higher and lower concentrations were inferior for growth.

Chemical Condition of Medium

No experiments on the inorganic requirements are to be described here as all of the media used contain sufficient inorganic constituents for all species (Lwoff, 1932).

In all media used the peptones, proteoses and proteins offered an adequate source of nitrogen. For the two species of *Tetrahymena* it was possible to test the relative effectiveness of various types of protein products on growth rate and maximum concentration. Various yeast products were tested as a substitute for and in combination with the standard proteose peptone. One per cent Difco yeast extract, one per cent filtered Yeast-Harris and 10 per cent liquid yeast autolysate were used. The rate of reproduction (as calculated by the formula

$$g = \frac{t \log 2}{\log b \log a},$$

where $g =$ the generation time and $t =$ the time in hours during which the population has been increasing, $a =$ the number of cells per unit volume at the beginning and $b =$ the number of cells at the end of time, t) in the case of *T. geleii,* was slightly lower in all three types of yeast media than in proteose peptone (Table I). The generation time for *T. vorax* was more than doubled (as compared with 2 per cent proteose peptone) in both yeast extract and yeast autolysate and was somewhat greater in Yeast-Harris (Table I). Some product of yeast autolysis (present in both the extract and the autolysate) seems to inhibit the reproduction of this species.

In the above experiments the various yeast factors were presented along with the yeast proteins. There remained the possibility that some

TABLE I

Tetrahymena. Comparison of growth in protein media. Average of four experiments.

Medium	Generation time in hours *T. geleii* (strain W)	*T. vorax*
1 per cent yeast extract	3.51	7.07
10 per cent liquid yeast autolysate	3.34	7.90
1 per cent Yeast-Harris (particulate)	3.65	4.61
2 per cent proteose peptone	2.78	3.54

of these factors might stimulate growth if more adequate protein products were present. Consequently a "yeast vitamin concentrate—Harris" which is practically free of native protein was added to a basic medium of proteose peptone. Various concentrations were tested on *Tetrahymena geleii* (strain W) and the results are given in Table II.

TABLE II

Tetrahymena geleii (strain W). Comparison of growth after the addition of various concentrations of Yeast Vitamin Concentrate (Harris) to a basic medium of 2 per cent proteose peptone plus 0.5 per cent dextrose. Average of two experiments.

Percentage Yeast Vitamin Conc.	Generation time	Population per ml. at end of log. phase	Maximum yield
	hours		*cells/ml.*
0	3.37	48000	310000
0.025	3.14	52000	330000
0.05	2.95	67000	380000
0.1	2.70	70000	400000
0.2	2.74	54000	365000

The reproductive rate increased with the concentration up to 0.1 per cent but was slightly lowered at 0.2 per cent. The addition of yeast concentrate consistently lowered the reproductive rate of *T. vorax.*

The addition of yeast vitamin concentrate to the particulate medium used for *Glaucoma* and *Colpidium* had no significant effect up to a concentration of 0.2 per cent although higher concentrations caused inhibition of growth. These observations are of little significance, however, as the basic medium must contain rather high concentrations of the yeast factors.

The addition of a separate source of carbon to the basic proteose peptone medium for *Tetrahymena* and the particulate medium for *Glaucoma* and *Colpidium* increased the length of the logarithmic phase and the maximum yield of all species except *T. vorax.* As was mentioned in the initial report on *T. vorax* (Kidder, Lilly and Claff, 1940) dextrose

TABLE III

Tetrahymena vorax. Comparison of growth after the addition of various concentrations of dextrose to a basic medium of 2 per cent proteose peptone. Average of four experiments.

Percentage dextrose	Generation time	Population per ml. at end of log. phase	Maximum yield
	hours		*cells/ml.*
0	3.55	10000	70000
0.5	4.60	7000	42000
1.0	5.01	5500	32000
2.0	9.56	890	6000

decreases the division rate in direct proportion to its concentration (Table III) and the maximum yield is lowered in the same manner.

Experiments were conducted to test the ability of the four species of ciliates to ferment some of the more common carbohydrates. One polysaccharide (Difco soluble starch), four disaccharides (Difco saccharose, Difco maltose, Difco lactose and Difco cellobiose), three monosaccharides (Difco dextrose, Difco levulose and Difco galactose) and two pentose sugars (Special Chemicals arabinose and Difco xylose) were used. To the two types of basic media 0.5 per cent of the above carbohydrates and 0.02 per cent brom thymol blue were added. These media were dispensed in Pyrex tubes and sterilization was accomplished in the autoclave at 15 pounds pressure for 12 minutes. After cooling each type of media was inoculated with the four species of ciliates and the results were noted by the color change of the indicator after 96 hours in the case of *Tetrahymena* and 240 hours in the case of *Glaucoma* and *Colpidium*.

TABLE IV

Fermentation of carbohydrates. All carbohydrates added to basic protein media in 0.5 per cent concentrations. Medium contained 0.02 per cent brom thymol blue. Six experiments.

	Organism			
Carbohydrate	*Tetrahymena geleii* (strain W)	*Tetrahymena vorax*	*Glaucoma scintillans*	*Colpidium campylum*
starch	+	+	+	−
sucrose	−	−	−	+
maltose	+	+	+	+
lactose	−	−	−	−
cellobiose	+	+	+	−
dextrose	+	+	+	+
levulose	+	+	+	+
galactose	−	−	−	−
arabinose	−	−	−	−
xylose	−	−	−	−

The results of these fermentation experiments are given in Table IV. *Colpidium* alone failed to utilize starch and cellobiose. On the other hand, *Colpidium* was able to utilize sucrose while the other three species were not. None of the ciliates fermented galactose although Colas-Belcour and Lwoff (1925) report fermentation of this monosaccharide by their strain of *Tetrahymena* (*Glaucoma piriformis*). Galactose, arabinose and xylose so inhibited the growth of all the species that these carbohydrates were not used in the quantitative growth studies. All of the other carbohydrates were re-investigated in growth flasks and the cultures followed by frequent counts. The indicator was omitted but otherwise the media were as above.

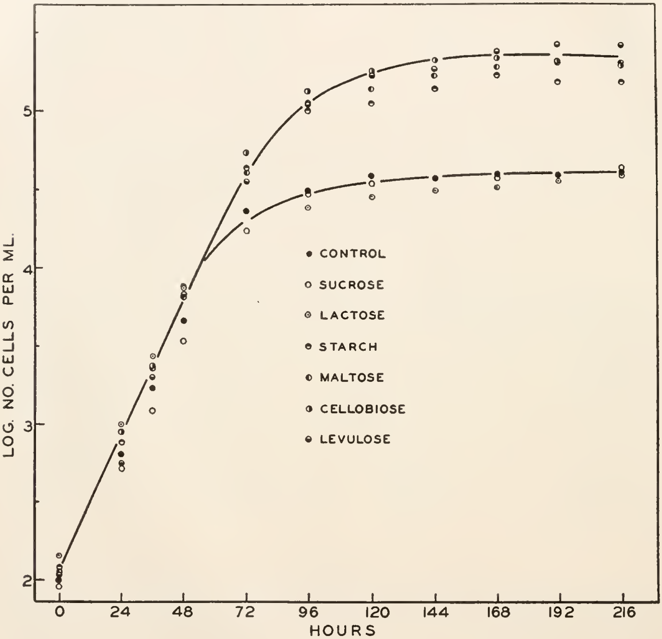


FIG. 5. *Glaucoma scintillans.* Effect on growth of the addition of carbohydrates to basic medium (1 per cent Yeast-Harris plus 2 per cent proteose peptone). All carbohydrates added in 0.5 per cent concentrations. Average of 3 experiments.

The division rate, length of logarithmic phase and maximum yield were slightly increased by all of the carbohydrates fermented by *Tetra-*

hymena gelcii (strain W). These increases were small but constant. No significant differences could be detected between any of the media containing fermentable carbohydrates. The acidity rose from an initial pH 6.8 to a final pH 4.8 in those flasks containing starch, maltose, cellobiose, dextrose or levulose while it fell in all others, including the control flasks, to pH 7.2.

Tetrahymena vorax, although it was able to ferment starch, maltose, cellobiose, dextrose and levulose (initial pH 6.8–final pH 5.4) was distinctly inhibited in its growth. Reproductive rate, length of logarithmic phase and maximum yield were decreased wherever fermentation occurred (see Table V for dextrose) and were unaffected (as compared with controls) in media containing carbohydrates which were not attacked (sucrose, lactose).

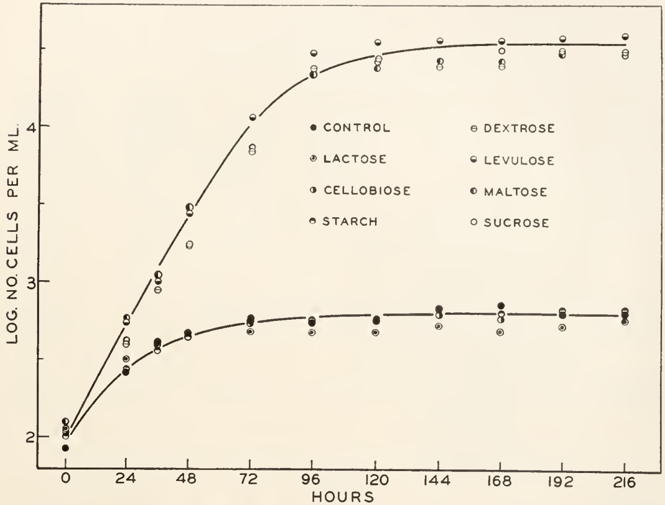


Fig. 6. *Colpidium campylum.* Effect on growth of the addition of carbohydrates to basic medium (1 per cent Yeast-Harris plus 2 per cent proteose peptone). All carbohydrates added in 0.5 per cent concentrations. Average of 5 experiments.

The most striking results of the addition of carbohydrates were found in the cases of *Glaucoma* and *Colpidium.* These results are given in Figs. 5 and 6.

The growth of *Colpidium* without carbohydrate was very slow and the maximum yield was low (not greater than 800 per ml.). The addition of 0.5 per cent dextrose, levulose, sucrose or maltose increased the rate of growth during the logarithmic phase and the maximum yield was increased to over 40,000 per ml. in some cases. For practical purposes, therefore, an additional source of carbon is a necessity for this ciliate.

The situation is somewhat different with *Glaucoma*. The division rate during the first 48 hours is not appreciably changed when a fermentable carbohydrate is added. Without a separate source of carbon, however, the end of the logarithmic phase is reached rather suddenly and the curve flattens, the concentration (about 42,000 per ml.) remaining relatively constant thereafter for many days. The addition of sucrose or lactose has no significant effect upon the cultures, but the addition of dextrose, levulose, maltose, cellobiose or starch causes an increase in the length of the logarithmic phase, a long phase of negative growth acceleration and a final yield in excess of 200,000 per ml.

TABLE V

Summary of growth characteristics.

Organism	Medium	Optimum pH	Generation time	Population at end of log. phase	Maximum concentration per ml.
			hours		
Tetrahymena geleii (W)	2 per cent proteose peptone, 0.5 per cent dextrose, 0.1 per cent Yeast Vitamin Conc.	5.6–8.0	2.69	58000	395000
Tetrahymena vorax	2 per cent proteose peptone	6.2–7.6	3.52	12000	110000
Glaucoma scintillans	1 per cent Yeast-Harris, 2 per cent proteose peptone, 0.5 dextrose	5.6–6.8	7.37	40000	270000
Colpidium campylum	"	5.4	11.56	7200	41000

Optimum pH

A number of experiments were conducted to determine the optimum pH limits for the four species of ciliates. For these experiments three types of media were used: 2 per cent proteose peptone for *Tetrahymena vorax;* 2 per cent proteose peptone plus 0.5 per cent dextrose for *T. geleii* (strain W); 1 per cent Yeast-Harris, 2 per cent proteose peptone, 0.5 per cent dextrose for *Glaucoma* and *Colpidium.* The pH was adjusted through a wide range of values (from pH 4.8 to pH 8.6) with HCl and NaOH.

The results of these experiments are contained in summary form in Table V.

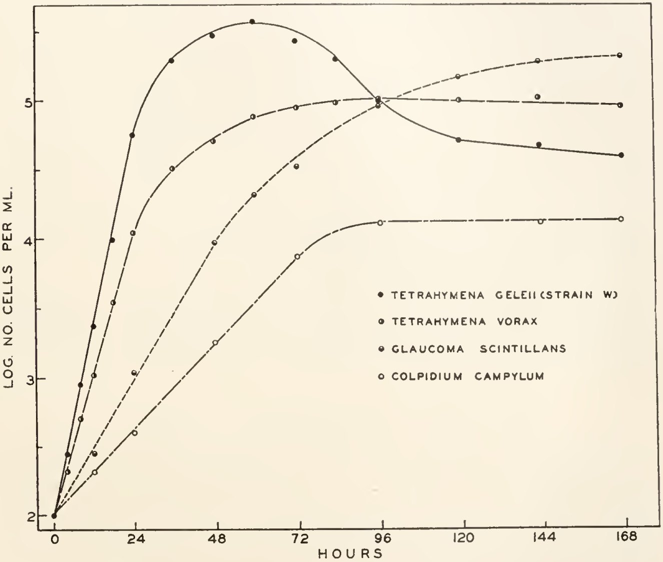


Fig. 7. Graphic comparison of the growth characteristics of four species of ciliates. Media as given in Table IX.

General Comparison of Growth Characteristics

Several interesting facts are brought out when the four species of ciliates are compared during the various phases of their growth. Figure 7 is a graph prepared from various experiments, each species growing under optimum conditions. A summary of data is given in Table V.

The growth rate of *Tetrahymena geleii* (strain W) is higher than any strain of ciliate in pure culture so far reported ($g = 2.69$ hrs.).

Unlike strain P (Phelps, 1935; 1936) and strain H (Kidder, 1941) of this species the negative acceleration period of strain W is quite long and the stationary phase is short. The concentration declines rapidly after the culture is approximately 60 hours old (initial inoculum of 100 cells per ml.) but the death rate decreases later (at about 120 hours) so that a concentration of 30,000–40,000 cells per ml. is maintained for many days.

Tetrahymena vorax grows at a regular rate ($g = 3.52$ hrs.) only during the first 24 hours. Thereafter a long negative growth acceleration phase ensues and the stationary phase is not reached until the culture is approximately 96 hours old. There is no decline in concentration, however, for many days so, in this respect, *T. vorax* resembles strains P and H of *T. geleii* (Phelps, 1935, 1936; Kidder, 1941).

In general the shape of the growth curve of *Glaucoma scintillans* is similar to that of *T. vorax*. The generation time during the logarithmic phase (first 48 hours of growth) is 7.37 hours. This rate gradually falls off and an extremely long negative growth acceleration phase takes place during which time the concentration increases from approximately 40,000 cells per ml. to over 200,000 per ml. The shape of this curve is reproducible under the conditions of these experiments.

The shape of the growth curve of *Colpidium campylum* is similar to that of strain H of *Tetrahymena geleii* (Kidder, 1941) although the growth rate is very low ($g = 11.56$ hrs.) as is the maximum yield (41,000 per ml.). This final concentration is maintained for many days.

Observations on Age and Size of Inoculum

As was stated earlier in this report, the ciliates used as inocula in the foregoing experiments were all taken from their logarithmic growth phases. Under these conditions no lag phase occurred. This corresponds to previous findings on controlled cultures (Phelps, 1935; Dewey and Kidder, 1940; Kidder, 1941). A lag phase invariably occurs if the ciliates which form the inoculum are taken from cultures which have passed the logarithmic growth phase. This statement holds for all four species used in the present study and has been reported for other strains and species (strains P and H of *Tetrahymena geleii*, Phelps, 1935; Kidder, 1941; *Perispira ovum*, Dewey and Kidder, 1940). The length of the lag phase increases, up to a certain point, in direct relation to the age of the parent culture.

The course of the growth is not dependent upon the size of the inoculum of *Tetrahymena geleii, T. vorax* or *Glaucoma scintillans*. Experiments on this point were conducted using large growth flasks (1 liter capacity) containing 500 ml. of media. Single ciliates were inoculated into these flasks. In order to insure the inoculation of active, single organisms they were first isolated into small containers made from the lower portion of shell vials. These containers had been previously placed in Petri dishes and sterilized. After the single ciliates had been isolated and checked under the dissecting binocular for number and activity, the whole container was lifted with sterile forceps and dropped into the culture flask. The initial inoculum is, in this case, 0.002 cells per ml. After growth has proceeded for sufficient time so that samples include enough cells for determination of numbers, the generation time is calculated and compared to the control flask which has received the usual inoculum of 100 cells per ml. No significant difference between the generation times in high and low inoculum cultures of the three species was obtained (Table VI). In no case did these single ciliates fail to establish perfectly normal cultures.

TABLE VI

Effect of size of inoculum. Figures represent generation time in hours. Volume of medium = 500 ml.

No. of cells per ml. inoculated	Organism			
	Tetrahymena geleii (strain W)	*Tetrahymena vorax*	*Glaucoma scintillans*	*Colpidium campylum*
100	2.69	3.52	7.37	11.66
0.002	2.71	3.48	7.41	18.25

Colpidium campylum did not give the same results (Table VI). In a number of the flasks inoculated with single ciliates no growth occurred. In those cultures which became established the generation time was significantly increased (18.25 hours as compared with 11.66 hours in the controls) and, what is more striking, the maximum yield was always very low (8,000 per ml. as compared with 40,000 per ml. in the controls). This species does not follow the same course as the other three and would seem to correspond to the reports of Robertson (1921–1927) on non-sterile organisms and of Mast and Pace (1938) on *Chilomonas*. Considering the general characteristics of *Colpidium*, however, I believe that there may be an explanation of the apparent "allelocatalytic" effect. Some substance or condition of the medium may be slightly detrimental to this ciliate. When large numbers of organisms are introduced no single cell receives a lethal amount of the toxic material. When a single ciliate is introduced into a large amount of medium it accumulates enough of the toxic substance to cause its death in some cases or to injure it in others. When the injury is sub-lethal it nevertheless permanently affects the cells. The lowering of the maximum yield in single-cell-inoculated cultures which become established seems to favor this hypothesis.

Discussion

No complete analysis of the protein requirements of any ciliate is available at the present time due to a number of factors. Lwoff (1932) and Lwoff and Lwoff (1937) have obtained some data on their strain of *Tetrahymena geleii* (*Glaucoma piriformis*) but until all of the supplementary factors in relation to nutrition are more perfectly known this knowledge must remain incomplete. Neither *Glaucoma scintillans* nor *Colpidium campylum* appears to offer satisfactory experimental material for studies along this line. They both require particles. The particles obtained from powdered yeast cells are of nearly unknown chemical constitution. About all we can say concerning these particles is that they are very complex. To these particles appear to be adsorbed the molecules of proteoses and peptones necessary for the optimum growth. An attempt was made to substitute animal charcoal (Norit) for the powdered yeast. *Colpidium* failed to ingest these particles and while *Glaucoma* did ingest them at first (black food vacuoles), they later refused to do so and very little growth resulted. Various other inert materials which were tried proved no more successful. There may well be other types of particles (such as precipitated proteins, etc.) which could be substituted but no appreciable advantage would be gained. Casein, a well-known protein, was used successfully to supply the particles but the growth was never as good as in the Yeast-Harris medium, even though a filtrate of the Yeast-Harris was added.

It appears strange that *Tetrahymena vorax* is inhibited by some factor in yeast while the reproduction of *T. geleii* (strain W) is accelerated. This situation is also true of the fermentable carbohydrates. No answer to the question of these specific differences is available at present. It will be interesting to compare the supplementary requirements of these two species with the yeast factor question in mind.

Living organisms as food have been found to be a necessity for a number of species of ciliates (Phelps, 1934; Kidder and Stuart, 1939; etc.). This is not the case with *Glaucoma* and *Colpidium*, however. The most apparent difference between growth on a favorable bacterium and in pure culture is rate of reproduction. The living bacteria accelerate growth. This is not true in the case of *Tetrahymena*, where no species of food organism tested was as favorable for growth as the dissolved protein materials.

The observations on the carbohydrates are interesting in showing specific differences in enzyme production. While all four species of ciliates used in this investigation produce an amylase and a maltase, none of them produce lactase. *Colpidium campylum* stands alone in producing invertase and failing to produce cellobiase. All species ferment dextrose and levulose and fail to ferment galactose, arabinose and xylose.

With the exception of galactose and the pentose sugars, the carbohydrates which were not fermented did not influence the growth of any of the ciliates, although Elliottt (1935) reports some cases where acceleration of growth resulted without acid fermentation. These cases, however, must be questioned as he calculated acceleration by yield after a given time (usually 72 hours). The reason for questioning the validity of this method has been given in a previous section of this report.

Galactose, arabinose and xylose were found to be inhibitory to all four species of ciliates. Elliott (1935) reports inhibition of *Tetrahymena geleii* (strains H and E) by galactose, while Colas-Belcour and Lwoff (1925) record the fermentation of galactose by their strain of *T. geleii* but give no data regarding growth.

In the experiments designed to test the effect of the initial pH of the medium upon the growth of the ciliates investigated there was no indication that two optima exist as was reported by Elliott (1933) for his strain of *Tetrahymena geleii.* In fact, there were no significant differences in generation time, length of logarithmic phase or maximum yield over a wide pH range in the case of *T. geleii* (strain W), *T. vorax* or *Glaucoma scintillans. Colpidium campylum* reproduces faster, for a greater length of time and to greater final concentrations when the pH of the medium is low (pH 5.4).

No data are available from these experiments as to the factors which limit the period of maximum reproductive rate or cause the death of the organisms during the later stages of the cultures. It should be pointed out that the growth characteristics given are valid only under the conditions outlined and might well be changed somewhat by varying these conditions. The accumulation of volatile products of metabolism, such as CO_2, or the reduction of O_2 tension could be largely overcome by aeration. Phelps (1936) found that aeration increased the length of the logarithmic phase of *Tetrahymena geleii* (strain P) but did not alter the generation time in the early stages of growth.

A point of some interest which should be brought out is what Elliott (1933) and Johnson (1935) called "acclimatization." These authors report the necessity for gradually reducing the number of bacteria in the process of sterilizing their ciliates (*Tetrahymena*). These observations were not confirmed on the ciliates used in this study. In every case establishment after complete sterilization followed immediately upon the presentation of an adequate medium. Another type of acclimatization was noted, however, in the case of *Glaucoma scintillans.* The growth rate (strain A) increased steadily during the first three months of sterile culture. The first calculations were based upon cursory data so this point was checked with strain B. One week after its initial isolation (May 27, 1940) growth flasks were inoculated and the generation time during the logarithmic phase was determined and found to be 12.21 hours. Cultures started June 10 grew more rapidly (generation time 10.64 hours) while those started on July 12 and September 20 were increasingly rapid (9.81 hours and 8.98 hours, respectively). Strain B, therefore, repeated what had been noted for strain A and although this strain does not reproduce as rapidly as strain A, even after four months, the same tendency of gradual adaptation to the sterile medium is shown.

SUMMARY

1. The growth characteristics of four species of holotrichous ciliates (*Tetrahymena geleii, T. vorax, Glaucoma scintillans* and *Colpidium campylum*), grown in pure culture, are given.

2. The two species of *Tetrahymena* are able to utilize dissolved nutritive materials while *Glaucoma* and *Colpidium* are dependent upon particulate materials in the media.

3. The growth of *T. geleii* is slightly accelerated by some factor in yeast and by the presence of fermentable carbohydrates (dextrose, levulose, maltose, cellobiose and starch) while inhibition of the growth of *T. vorax* results when these materials are present.

4. The maximum yield of *Glaucoma* and *Colpidium* is greatly increased by fermentable carbohydrates.

5. *Colpidium* fails to ferment cellobiose but, unlike the other three species, does ferment sucrose.

6. Galactose, arabinose and xylose, while not fermented by any of the four species of ciliates, inhibit the growth of all.

7. The optimum range of pH values for *T. geleii* (strain W) is wide (pH 5.6 — pH 8.0) ; *T. vorax* is slightly more limited (pH 6.2 — pH 7.6) ; *Glaucoma* is limited to the acid range (pH 5.6 — pH 6.8), while *Colpidium* grows best at pH 5.4.

8. In the cases of *Tetrahymena geleii, T. vorax,* and *Glaucoma scintillans* when single ciliates from the logarithmic growth phase are inoculated into 500 ml. of media (initial inoculum = 0.002 cells per ml.) there is no lag phase and the generation time is not reduced (as compared with controls).

9. Single *Colpidium campylum* inoculated into 500 ml. of media often die. When a culture is established the generation time is longer and the maximum yield is smaller than when many cells are inoculated. It is suggested that these results are correlated with slight toxicity of the medium.

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THE EFFECT OF SAPONIN ON THE OSMOTIC HEMOLYSIS OF CHICKEN ERYTHROCYTES[1]

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I

Although the hemolysis of erythrocytes by lytic agents such as saponin has received much attention, few investigators have studied the effect of these agents prior to hemolysis. Ponder (1937) reported a decrease in the fragility of rabbit erythrocytes which had been exposed to sub-lytic concentrations of lysins. This effect, he believed, was similar to the action of narcotics (cf. Jacobs and Parpart, 1932). Davson and Danielli (1938) reported that saponin caused no loss of K^+ from erythrocytes in either sub-hemolytic or hemolytic concentrations.

The present experiments were performed to determine what effect saponin would have on the penetration of small molecules prior to the time that the membrane became permeable to the hemoglobin molecule.

II

Chicken blood was obtained by cardiac puncture and then defibrinated. One half a cc. of blood suspended in 5 cc. of Ringer-Locke required approximately 0.2 cc. of 1 per cent saponin in Ringer-Locke to cause slight hemolysis in two hours. A control suspension was similarly prepared, omitting the saponin. The slight difference in total volume (0.2 cc. in 5.5 cc.) did not introduce any error, since the method used to measure permeability is not influenced by variations in the number of cells within a wide range.

In this way stock suspensions of control and experimental cells were prepared. In most experiments 0.2 cc. of these suspensions were added to 10 cc. of an isosmotic solution of the substance whose rate of penetration was to be measured. Permeability, fragility, and swelling measurements were made at a temperature of 37° ± 0.5° C., using the photronic cell technique usually employed in this laboratory (cf. Hunter, 1936; Hunter and Pahigian, 1940). In every experiment sufficient

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[1] One of the authors (F. R. H.) is indebted to the American Association for the Advancement of Science for a grant-in-aid.

$NaHCO_3$ was added to insure complete clearing of the suspension (Stringer et al., 1940; Hunter et al., 1940). Permeability measurements were made until the experimental cells were found to hemolyze more rapidly than the controls when placed in the isosmotic solutions of penetrating substances. At this time the experimental solution was diluted with Ringer-Locke and centrifuged. In some of the experiments the supernatant fluid after centrifugalization was colorless and all of the packed cells were red, which indicated that the saponin had not caused any hemolysis. In others, the supernatant fluid was red and some of the cells were white. This indicated that the saponin had been allowed sufficient time to destroy the membranes of some of the cells and liberate the hemoglobin. Experiments in which this had happened served to indicate that the cells whose permeability was being studied had been exposed to hemolytic concentrations of the lysin, but the process had been stopped by removing the lytic agent before many of the cells had been hemolyzed. After the cells had been centrifuged, the supernatant fluid containing the saponin was discarded and the cells were resuspended in a volume of Ringer-Locke sufficient to give a suspension containing approximately the same number of cells per unit volume as the control. Additional washing with Ringer-Locke was unnecessary, as these cells would remain unhemolyzed for several days. These resuspended cells exhibited the same permeable properties that they had had immediately preceding the centrifugalization. The control cells were not centrifuged in every experiment, since this treatment had no marked effect on their permeability.

Some of the molecules penetrated the cells so rapidly at 37° C. that measurements could not be made using the photronic cell technique. In these cases the rate of hemolysis was measured by eye at room temperature (about 23° C.). The complete hemolysis curve could not be obtained in this way, but it was possible to make comparisons by measuring the times for a given percentage of the cells to hemolyze.

Permeability measurements were begun as soon as the control and experimental suspensions were prepared. A comparison of the effect of saponin on the permeability to glycerol and monoacetin was made using the photronic cell apparatus, while comparisons using the other method were made between various rapidly penetrating lipoid-soluble and insoluble molecules. Since it usually required at least one hour before the saponin produced any marked effect, there was sufficient time to make several measurements before the cells were centrifuged.

After the suspensions had been centrifuged and the cells resuspended in Ringer-Locke, hemolysis measurements were made using a number of different substances. Centrifuged cells were used to obtain the swelling and fragility data. In some experiments, higher concentrations of saponin were used which produced slight hemolysis in 10–15 minutes. Cells obtained in this way gave the same results as those which had been exposed to lower concentrations of the lysin.

III

The data have been analyzed in several ways. In Table I are listed the average number of seconds required for 80 per cent hemolysis (one-half the total deflection) of the experimental and the control cells when placed in isosmotic solutions of the various substances studied. Since the change in hemolysis time depends on a number of factors such as the concentration of saponin, and the time allowed for its action, there is great variability observed in this value. To reduce this variability, only the figures obtained after the cells had been centrifuged are recorded. The differences in the percentage change which remain result from the fact that the saponin acted for a longer time in some experiments than in others. The table merely indicates that the permeability to a number of different substances has been greatly increased. The data for those substances which are starred were obtained from measurements made by eye. Figure 1 presents a representative control and experimental curve.

TABLE I

The effect of saponin on the osmotic hemolysis of chicken erythrocytes.

Substance	Average time in seconds for 80% hemolysis		Per cent change	Number of observations
	Control	Experimental		
*Water	18	7	−61.1	1
*Ethylene glycol	47	38	−19.2	10
*Diethylene glycol	75	39	−48.0	3
Triethylene glycol	130	87	−33.1	8
Urea	148	112	−24.3	6
Thiourea	193	148	−23.3	7
Glycerol	193	104	−46.1	38
Malonamide	868	273	−68.5	11
Erythritol	1125	292	−74.0	1
*Diacetin	34	23	−32.3	5
Monoacetin	144	63	−56.3	31
*Acetamide	38	22	−42.1	3
*Propionamide	38	22	−42.1	5

* Measurements made by eye.

Having established the fact that treatment with saponin could increase the permeability of the cell membrane without causing hemolysis, the data were analyzed in an attempt to demonstrate any differential effects. The possibility that an increase in permeability to lipoid soluble-molecules might appear first was considered. These data are presented in Table II. Once again there is considerable variability, but the evidence indicates that the experimental treatment increases the permeability to lipoid-soluble and lipoid-insoluble molecules similarly. The time at which the increase is first noted and the degree of change are the same for the two types of substances.

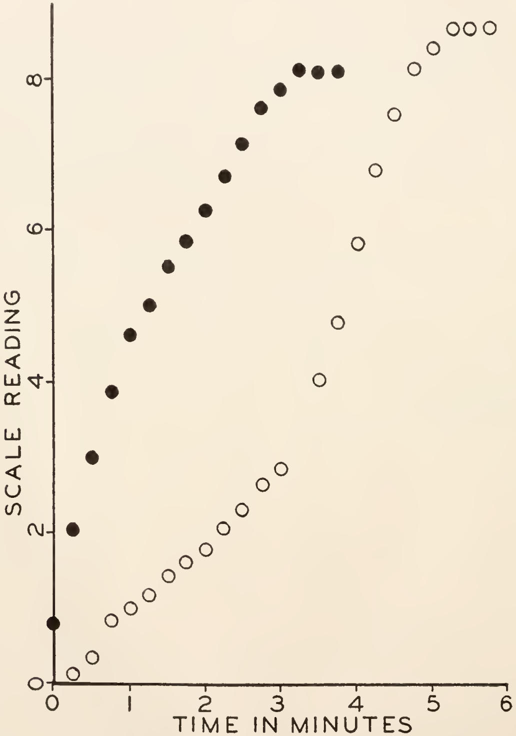

Fig. 1. The effect of saponin on the osmotic hemolysis of chicken erythrocytes in glycerol. ○—control; ●—experimental. (Scale reading represents degree of hemolysis.)

Although the increase in permeability to lipoid-soluble and lipoid-insoluble molecules apparently occurs at the same time, it was thought

TABLE II

Data comparing the effect of saponin on the osmotic hemolysis of chicken erythrocytes in lipoid-soluble and lipoid-insoluble molecules after a short exposure to the saponin.

	Percentage change			
Date	Glycerol	Monoacetin	Ethylene glycol	Diacetin
3/6/40	−42.2	−62.2		
3/8/40	−13.8	−22.7		
3/27/40			− 7.2	−15.2
3/29/40			−18.3	−12.3
3/30/40	−11.1	− 4.5	+ 0.5	− 2.4
4/6/40			−16.2	−20.9
4/8/40	−18.0	− 9.5		

TABLE III

Data comparing the effect of saponin on the osmotic hemolysis of chicken erythrocytes in lipoid-soluble and lipoid-insoluble molecules after a long exposure to the saponin.

Glycerol			Monoacetin		
Time in seconds for 80% hemolysis		Percentage change	Time in seconds for 80% hemolysis		Percentage change
Control	Experimental		Control	Experimental	
200	50	−75.0	205	110	−46.3
215	142	−33.9	205	150	−26.8
175	24	−86.3	175	26	−85.2
195	115	−41.0	195	95	−51.3
195	95	−51.3	195	68	−65.1
131	18	−86.3	175	20	−88.6
125	50	−60.0	85	50	−41.2
106	80	−24.5	140	28	−80.0
165	100	−39.4	110	95	−13.6
154	152	− 1.3	130	112	−13.8
255	60	−76.5	155	38	−75.5
210	40	−81.0	130	90	−30.8
185	140	−24.2	130	40	−69.2
185	55	−70.3	130	110	−15.4
237	157	−33.8	175	25	−85.7
237	80	−66.2	205	135	−34.1
237	25	−89.5	205	40	−80.5
237	35	−85.2	205	23	−88.8
237	135	−43.0	146	82	−43.8
237	188	−20.7	146	130	−11.0
237	120	−49.4	146	70	−52.0
215	35	−83.7	127	13	−89.8
215	26	−87.9	127	13	−89.8
215	130	−39.5	127	37	−70.9
Average 192	86	−55.2	157	67	−57.3

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possible that after the membrane had become considerably altered a difference in rate of penetration might appear. The data in Table III are presented as evidence on this point. These were obtained after the saponin had completed its action on the cell and had been removed by centrifugalization. The results indicate that under similar treatment the permeability to a lipoid-soluble molecule such as monoacetin is increased the same amount as the permeability to a lipoid-insoluble molecule such as glycerol.

Table IV

Data comparing the effect of saponin on the rate of penetration of a small and a large lipoid-insoluble molecule.

Glycerol			Malonamide		
Time in seconds for 80% hemolysis		Percentage change	Time in seconds for 80% hemolysis		Percentage change
Control	Experimental		Control	Experimental	
131	18	−86.3	670	204	−69.6
125	50	−60.0	578	126	−78.2
165	100	−39.4	805	644	−20.0
154	152	− 1.3	729	675	− 7.4
255	60	−76.5	1080	180	−83.3
237	157	−33.8	1080	75	−93.1
237	80	−66.2	920	540	−41.3
237	25	−89.4	920	210	−77.2
237	35	−85.2	920	83	−91.0
237	135	−43.0	920	110	−88.0
237	120	−49.4	920	150	−83.7
200	110	−45.0	730	390	−46.6
230	110	−52.2	810	330	−59.3
190	35	−81.6	570	60	−89.5
180	65	−63.9	480	65	−86.5
330	320	− 3.0	1190	1070	−10.1
340	315	− 7.4	1260	1080	−14.3
330	285	−13.6	630	270	−57.1
330	260	−21.2	690	210	−69.6
125	45	−64.0	400	120	−70.0
125	45	−64.0	640	150	−76.6
Average 221	120	−45.7	807	321	−60.2

Finally, a comparison was made of the effect of the experimental treatment on the penetration of a large molecule such as malonamide and a small molecule such as glycerol. In this case there appeared to be a consistent difference, but in order to be certain, an additional series of experiments was performed. All of the data are included in Table IV. The last ten sets of readings were from the second series of experiments. These data indicate that the rate of entrance of malonamide is increased more by the experimental treatment than the rate of entrance of glycerol.

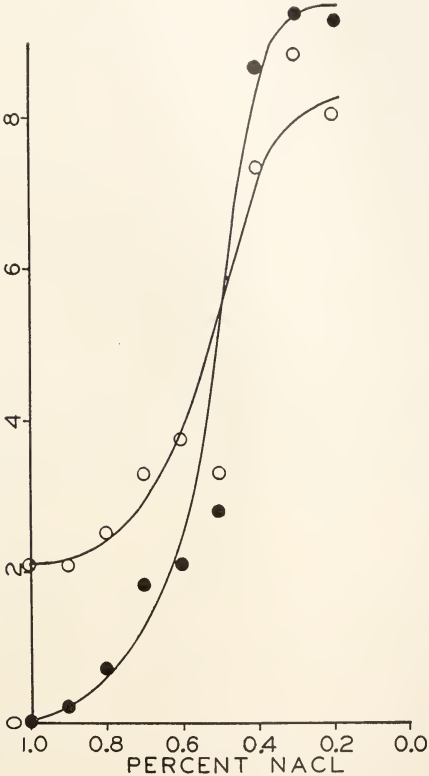

Fig. 2. The effect of saponin on the fragility of chicken erythrocytes. ○—control; ●—experimental (Scale reading represents degree of hemolysis.)

A decrease in the time for hemolysis does not necessarily indicate an increase in permeability (cf. Hunter and Pahigian, 1940). In order to test for a possible change in cell fragility, the following experiments were performed. Twenty cu.mm. of blood were added to 10 cc. of NaCl solutions of concentrations from 1.0 per cent to 0.2 per cent. The pH of these solutions was adjusted by the addition of two drops of $NaHCO_3$ to each tube. A typical pair of curves is presented in Fig. 2. These data indicate that there is little, if any, change in the volume at which the experimental cells hemolyze.

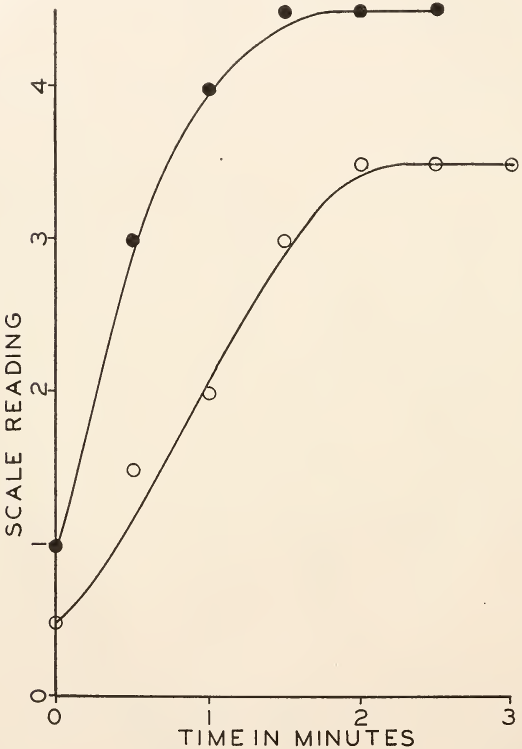

Fig. 3. The effect of saponin on the rate of swelling of chicken erythrocytes. ○—control; ●—experimental. (Scale reading represents amount of swelling.)

As a final test, swelling experiments were performed. Figure 3 presents a typical pair of curves obtained when control and experimental cells were added to an hyperosmotic solution of 0.3 M glycerol in Ringer-Locke. It can be seen that the experimental cells swell more rapidly than the controls. These data, then, definitely indicate an increase in permeability resulting from the experimental treatment.

IV

Schulman and Rideal (1937) presented evidence which indicated that the lytic action of saponin resulted from its reaction with cholesterol in the cell membrane. This would suggest that the experimental cells in the present investigation had altered membranes as a result of the action of saponin on some portion of the membrane in which lipoids were involved. The fact that the rate of penetration of lipoid-insoluble molecules, as well as that of lipoid-soluble molecules, was increased, would indicate that lipoid molecules in the membrane were in some way associated with the channels through which both types of molecules pass. A recent series of experiments by Ballentine and Parpart (1940), in which the effect of lipase on the cell surface was investigated, gave similar results. By making chemical analyses, these authors concluded that the lipase split fatty acids from the phospholipids in the cell surface. They suggested that these phospholipids were " an important structural unit in determining the rate of penetration in the aqueous channels."

As a result of the experiments in which lipase was used and those in which saponin was used, there is evidence to indicate that some of the lipoids in the cell membrane influence the passage not only of lipoid-soluble molecules but also of lipoid-insoluble molecules. It has been demonstrated that phospholipids are one type of molecule involved and the data contained in the present investigation suggest that cholesterol may be another.

SUMMARY

1. Chicken erythrocytes exposed to low concentrations of saponin have their membranes altered.
2. By removing the saponin and resuspending the cells in Ringer-Locke, they will remain unhemolyzed for several days, even though the membranes have been altered.
3. These cells are more permeable to both lipoid-soluble and lipoid-insoluble molecules.
4. The penetration of both types of molecules appears to be affected equally.
5. The rate of penetration of a large molecule such as malonamide is increased more by this treatment than the rate of penetration of a smaller molecule such as glycerol.
6. The fragility of these cells is not increased by this treatment.

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THE ROLE OF TISSUES IN THE ANAEROBIC METABOLISM OF THE MUSSEL ANODONTA HALLENBECKII LEA

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INTRODUCTION

Lamellibranch mollusks possess the capacity for enduring anaerobiosis for a considerable time. Such a capacity is advantageous to tidal zone forms which are exposed to air at low tide, and likewise to freshwater mollusks which may have to endure low oxygen content of polluted water as well as exposure. Recognition of this peculiar ability has led to investigation of anaerobic metabolic processes of mussels. If the stream of water passing over the gills is cut off, the oxygen supply fails while carbon dioxide accumulates. The manner in which the mollusk deals with accumulating carbon dioxide has been the subject of several investigations.

Collip (1921) showed that the marine form *Mya arenaria* used calcium to buffer carbon dioxide. Dotterweich and Ellsner (1935) showed that in the freshwater mussel *Anodonta cygnea* most of the carbon dioxide formed during anaerobiosis entered into combination with calcium to form calcium bicarbonate. A small amount was buffered by calcium proteinate. They concluded that in general calcium in the shell of mollusks may be utilized as an alkali reserve.

Recent investigation by Dugal and Irving (1937) indicated that tissues as well as body fluids are involved in adjustment to oxygen lack. Mantle tissue of *Venus mercenaria* was found to accumulate carbon dioxide and calcium just as did mantle cavity fluid.

The work reported in the present paper was an investigation of the adjustment of a freshwater mollusk to a disturbance of the acid-base balance resulting from anaerobiosis. Particular reference was made to the rôle of mantle and gill tissues in this adjustment. Determinations of the carbon dioxide content gave results which indicated that mussel tissues were able to buffer carbon dioxide. The relation of calcium to the buffering process was studied. Observations were made on the oxygen consumption of tissues taken from asphyxiated animals. Evidence of an oxygen debt was found, showing that dissimilative processes were continuing through the period of anaerobiosis.

Materials and Methods

Animals used were freshwater mussels taken in the vicinity of Durham, N. C. They were identified by Dr. Henry van der Schalie of the University of Michigan Museum of Zoölogy as *Anodonta hallenbeckii* Lea.

Control animals were kept in tanks of running water. In this situation the valves remained open, allowing a constant stream of water to pass over the gills. Experimental animals were removed from such tanks and placed in a refrigerator with an air temperature of 6 to 8° C. At this temperature clams survived about a month. When removed from water *Anodonta* closed the shell valves. In this position exchange of gases between animal and environment was impossible. Any opening of the shell was accompanied by leakage of fluids from the mantle cavity. Leaking animals were not included in the experiments.

Tissues used were gill, mantle, and kidney tissue. Some observations were made on pallial muscle and foot muscle.

The rate of oxygen consumption of gill, mantle, and kidney tissue was measured in a standard Warburg apparatus. Tissue samples weighing about 0.1 gram were suspended in a salt solution containing 0.153 per cent NaCl. Absorption of carbon dioxide was accomplished with 20 per cent KOH. The temperature was held at 25° C. Measurements were made over a period of sixty minutes.

Carbon dioxide content of gill and mantle was determined by an adaptation of the Van Slyke manometric method for the determination of blood gases. The gas burette of the apparatus was modified from that described by Ferguson and Irving (1929). A ground joint at the lower end of the extraction chamber allowed the introduction of tissue. A weighed sample of tissue was placed in the extraction chamber, the burette put in place, and the joint made secure. Carbon dioxide was liberated by 0.1 N HCl introduced through the upper stopcock. Usually complete extraction required 45 minutes of shaking. Carbon dioxide was absorbed with air-free 1.5 N NaOH. The values P_1 and P_2 and the correction factor, c, were determined in the usual way.

Conversion of the observed pressure of carbon dioxide into cubic centimeters of gas was made according to the formulae modified for use with tissue samples by Ferguson and Irving (1929). Values for specific gravity were necessary for the conversion formulae. These values as determined were: for mantle, 1.04; for gill, 1.12.

Care was taken to maintain constancy in the method of obtaining and weighing tissue samples. It is felt that the values for carbon dioxide content are comparable, although they may not be absolute.

Calcium content of clam tissues was determined from samples digested in a mixture of concentrated nitric and perchloric acids. Calcium was precipitated from the digest as oxalate, redissolved and titrated with permanganate.

RESULTS

Oxygen Consumption

Respiration of tissues from aerobic and anaerobic animals was compared. The results are given in Table I. The values for control animals are based on four determinations. They agreed closely. There were wider differences in the determinations on tissues from anaerobic animals. These values have been arranged by length of anaerobic period, and also averaged into one value for asphyxiated animals.

TABLE I

Oxygen uptake of gill, mantle, and kidney tissue. Values are averages of two to four determinations and represent cubic millimeters of oxygen consumed per hour per milligram dry weight of tissue at 25° C.

Days out of water	Kidney	Mantle	Gill
14	4.70	1.32	.486
10	3.66	1.34	.766
8	2.69	1.24	.652
6	2.82	1.45	.758
4	3.24	1.02	.660
2	2.82		.573
Average	3.32	1.27	.649
Average of controls	2.11	1.02	.421

Tissues removed from asphyxiated animals consumed more oxygen per hour per unit weight than did tissues from control animals. This was true for the first hour after removal. Determinations were not carried beyond this point. It is therefore impossible to make any calculation of the total extra oxygen required. However, the increase noted suggests the paying off of an oxygen debt incurred during anaerobiosis.

The respiration rates are referred to dry weight of tissue. It was found that mussel tissues varied in water content from one individual to another. There was no evidence of a correlation between dry weight and anaerobic period. The observed percentages dry weight as averaged from a large number of samples studied are given below:

mantle	3.9
kidney	8.6
gill	24.0

It is interesting that the rate of oxygen consumption of kidney tissue was much higher than that of other tissues studied. According to Holmes (1937), the high rate of respiration of mammalian kidney tissue is due to osmotic work done by excretory cells. Probably a similar explanation fits the case of mussel kidney.

Carbon Dioxide Content

Results of the determination of the carbon dioxide content of gill and mantle are given in Table II. The following points are to be noted:

TABLE II

Carbon dioxide content of mantle and gill. Values are expressed as cubic centimeters of gas at standard temperature and pressure and equivalents of carbon dioxide in one hundred grams fresh tissue. Averages of several determinations are represented.

Days out of water	Mantle	Gill	Mantle	Gill
	cc./100 gr.	*cc./100 gr.*	*equiv./100 gr.*	*equiv./100 gr.*
0	25.0	322	0.0022	0.0287
1		399		0.0356
2	32.2	372	0.0028	0.0332
3	35.0	369	0.0030	0.0328
4	32.0	405	0.0028	0.0376
6	34.4	430	0.0030	0.0392
8	43.6	455	0.0038	0.0406
10	44.2	487	0.0038	0.0432
12		499		0.0444
14	47.7	512	0.0042	0.0456

1. Gills contained approximately ten times as much carbon dioxide as did mantles.

2. There was a steady increase in the amount of carbon dioxide accumulating in gill tissue during anaerobiosis.

3. Carbon dioxide accumulated in mantle tissue in proportion to the increase in gill tissue. The equivalents of carbon dioxide in mantle doubled during asphyxiation.

For purposes of comparison with the amount of calcium present, the values for carbon dioxide were converted into equivalents and are also given in Table II.

Calcium Content

It was found that the calcium content of the tissues studied did not vary significantly with the period of anaerobiosis. Averages from a large number of determinations are given below, expressed as milligrams of calcium per gram dry weight of tissue.

foot muscle	8	mg./gram tissue
pallial muscle	31	"
kidney	46	"
mantle	62	"
gill	175	"

Gill tissue contained a large amount of calcium as compared with other tissues. This may be correlated with the relatively high dry weight of gill tissue. The small amount of calcium found in foot muscle is surprising when considered with the other values.

By using the percentage dry weight of mantle and gill tissue it was possible to calculate equivalents of calcium per one hundred grams fresh tissue. These were found to be: for mantle, 0.0045; for gill, 0.21.

Discussion

Study of the functioning of animal tissue in buffering processes has not been investigated in many species. Dotterweich (1933) showed that the calciferous glands of earthworms were capable of giving up calcium to buffer carbon dioxide accumulating in body fluids. Banus and Katz (1927) found weak buffering by hind leg muscles of a cat. A similar effect was noted by Irving and Chute (1932) in muscle.

A buffer system in the tissues of *Anodonta* is indicated by a study of the carbon dioxide and calcium content of certain tissues. Gill tissue seems to be most active in this respect.

From the data given above, it is seen that one hundred grams fresh gill tissue contain 0.21 equivalents of calcium, and 0.0287 equivalents of carbon dioxide (see Table II). This proportion indicates that most, possibly all, the calcium is present in some form other than carbonate.

During anaerobiosis the carbon dioxide level rises, increasing to 0.0456 equivalents at 14 days. This increase is not accompanied by an increase in the hydrogen ion concentration. The hydrogen ion concentration of the tissue was measured colorimetrically, and was found to vary less than 0.05 from pH 6.8 for gill, 6.9 for mantle. Apparently the accumulating carbon dioxide is bound in some way so that an increase in hydrogen ions does not occur.

It was suggested by Dotterweich and Ellsner (1935) that a calcium-proteinate might act as an additional buffer in the fluid of *Anodonta cygnea*. In that system calcium carbonate was the principal alkali reserve. In the tissues of *Anodonta hallenbeckii* it would seem that calcium-proteinate, or some other combination of a weak acid with calcium, is the chief buffer, with the carbonate playing at the most a minor rôle.

In the case of mantle tissue 0.0045 equivalents of calcium are present in the normal mantle. Carbon dioxide increases from 0.0022 equivalents in the normal tissue to 0.0042 equivalents in the asphyxiated tissue. The calcium and carbon dioxide are then in a one-to-one ratio. This would indicate a more limited calcium reserve in mantle than in gill.

Dugal (1939) has shown that in *Venus* the calcium reserve may be augmented by calcium from the shell. Tissues of *Anodonta* maintain a steady calcium level.

Calcium is not only the chief component of the hard parts of mollusks but also forms a considerable portion of the alkali reserve. The same factors which govern the precipitation of solid calcium in the shell are responsible for the deposition of calcium in tissues. It is a point of interest that freshwater clams possess large deposits of calcium in their gills, and marine clams possess the larger deposits in mantle tissues (McCance and Shipp, 1933). There may be some correlation here with the fact that glochidia develop in the gill pouches of freshwater mussels and may derive calcium for their shells from the abundant supply available.

Jatzenko (1928) showed that certain freshwater mussels build up an oxygen debt during anaerobiosis. It is to be expected that individual tissues would also show such a debt. All activity does not cease when the clam is temporarily asphyxiated. Some of it continues. Ciliary action such as accounts for a great deal of the oxygen consumption of gill and mantle probably does decrease to some extent. Osmotic work which is characteristic of kidney tissue continues and may even increase during anaerobiosis. Data for individual tissues as presented in Table I show that oxygen consumption of mussel tissues is higher immediately after a period of asphyxiation than under normal conditions.

The source of energy for activities carried on during anaerobic periods is generally laid to a glycolytic process. However, there has as yet been no isolation of the tissue or tissues mainly responsible for the glycogen reserve. The problem of the energy source and its localization is a pertinent one to a complete explanation of the anaerobic metabolism of mussels.

SUMMARY

Tissues of *Anodonta hallenbeckii* are capable of buffering carbon dioxide accumulating during anaerobiosis. Calcium compounds present in gill and mantle serve as an alkali reserve. During anaerobiosis carbon dioxide increased in the tissues studied while the hydrogen ion concentration remained constant. It is concluded that accumulated carbon dioxide was buffered by calcium present.

Gills contain large amounts of calcium which is present in some form other than carbonate.

Kidney tissue showed a very high rate of respiration. Mantle and gill showed low rates. After anaerobic periods the rate of respiration showed a tendency to increase. This may be taken as evidence that these tissues continued to do work during anaerobiosis.

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THE EFFECT OF THE CIRCULATION OF WATER ON THE DISTRIBUTION OF THE CALANOID COMMUNITY IN THE GULF OF MAINE[1]

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Damas (1905) has pointed out that the flow of water tends to dissipate local populations of pelagic organisms, and that the permanence of breeding stocks may be maintained by the existence of eddies. His predictions have been strikingly confirmed by hydrographic observations in the Norwegian Sea (Sømme, 1933). Along the margins of the Gulf of Maine the permanence of the stock of *Sagitta elegans* is correlated with the stability of the hydrographic conditions which exist in different regions (Redfield and Beale, 1940). Walford (1938) has indicated the importance of fluctuations in the circulation on Georges Bank to the fate of haddock eggs spawned in that region. These studies and that of the author (1939) on the population of *Limacina retroversa* emphasize the rapidity with which currents move pelagic organisms about within the Gulf. It becomes a problem whether the community of the basin of the Gulf is truly endemic, and by what mechanism a breeding stock is maintained within the Gulf. Sømme (1934) has discussed this question in regard to the copepod population of the Lofoten area.

Bigelow (1926), who has described the zoöplankton of the Gulf in great detail, considers that the species which form the bulk of the pelagic population are endemic in origin, breeding with sufficient regularity and abundance to maintain the local stock by local reproduction. From its dominating member, *Calanus finmarchicus,* he has referred to the population as the calanoid community.

We have measured the catches taken in the Gulf during a year-round survey and will attempt to explain the distribution of numerical abundance in terms of the pattern of currents obtained during the period of observation.

DATA

The data employed in the present study were collected in the course of cruises made by the research vessel "Atlantis" during the years of 1933 and 1934. The dates of these cruises and the numbers of the stations occupied are given in Table I. Thirteen cruises were made in the course of fifteen months, with the result that 684 hydrographic stations in the Gulf of Maine and its adjacent waters were occupied. At no time did a period longer than two months elapse without observation. The routine hydrographic and chemical data are published in the Bulletin Hydrographique (1933, 1934).

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[1] Contribution No. 281 from the Woods Hole Oceanographic Institution.

A supplementary cruise was made in May, 1936 in order to confirm certain observations made during the primary survey.

In the course of the cruises standard vertical hauls were made with a 1.5 meter Heligoland net of No. 0 silk having 38 meshes to the inch (Künne, 1933). The net was hauled from a point near the bottom to the surface at all stations occupied, weather permitting. This type of haul was selected in preference to the oblique haul in the belief that the procedure could be carried out uniformly as a part of the routine duties of the ship's company. Unfortunately, it proved impossible to use the net in rough weather, so that data are lacking from many stations, particularly those made in the winter months. The yields of the successful hauls have been measured by collecting the plankton on filter paper in a Buchner funnel. Suction was continued until the preserving fluid ceased to flow, whereupon the "dry" plankton was introduced into a measured volume of fluid and the resulting increase in volume noted. The data so obtained were reduced to figures expressing the number of cubic centimeters of "dry" plankton under each square meter of the sea surface. Before filtering and measuring collections, any large gelatinous organisms were removed (*Salpa,* ctenophores, medusae) with the result that the measurements reflect primarily the abundance of the crustacean community.

TABLE I

Cruise No.	Dates	Stations	Number
16 and 17	June 19–July 10, 1933	1643–1721	79
21	Sept. 2–Sept. 14, 1933	1741–1802	62
22	Oct. 17–Oct. 29, 1933	1803–1860	58
23	Dec. 2–Dec. 11, 1933	1861–1906	46
24	Jan. 8–Jan. 13, 1934	1907–1934	28
26	Mar. 21–Mar. 29, 1934	2019–2070	52
27 and 28	Apr. 17–May 13, 1934	2071–2164	94
29	May 21–June 3, 1934	2165–2215	51
31	June 25–July 1, 1934	2217–2236	20
34	Aug. 10–Aug. 11, 1934	2252–2259	8
37	Sept. 17–Sept. 27, 1934	2268–2303	36
55	May 14–May 19, 1936	2555–2583	29

Since most of the hauls with which we are concerned were made in

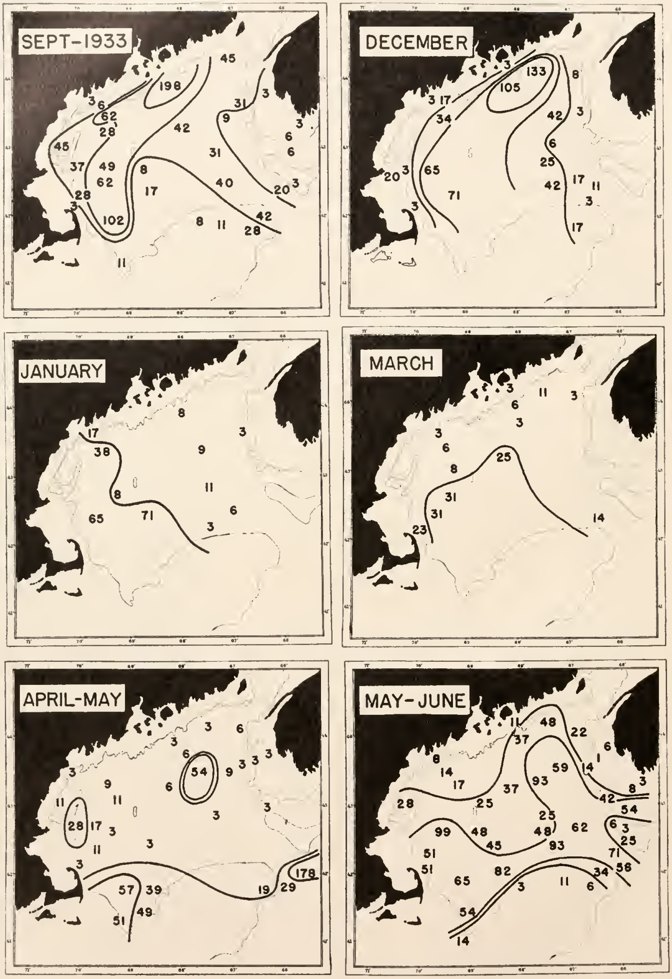

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FIG. 1. Volumes of zoöplankton taken in vertical hauls between September, 1933 and June, 1934. Numbers represent the cubic centimeters taken per square meter of sea surface. Contour interval 25, 50, 100, and 200 cc. per square meter.

depths greater than 100 meters, above which level most of the population may be expected to occur, these figures are thought to express the density of population more precisely than numbers reduced to unit volume of water strained. The general character of the results is not altered by expressing the catch in terms of the yield per cubic meter.

The determination of the "dry" volume of the catch by the method of filtration and displacement yields smaller values than are obtained by the "wet" method of allowing the animals to settle in a calibrated container. In order that our results may be compared with those of Bigelow and others who employed the settling method, a number of samples have been measured by both methods. The wet method gave values on the average 4.9 times higher than the dry method, the ratios varying between 3.3 and 7. The ratio was smaller in the case of the larger samples, due perhaps to the tighter packing of large samples in the wet method and to the greater retention of water when large quantities of organisms are filtered off in the "dry" method.

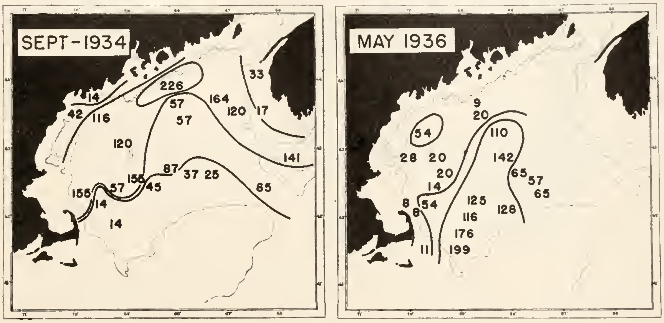

Fig. 2. Volumes of zoöplankton taken in vertical hauls in September, 1934 and May, 1936. Numbers represent the cubic centimeters taken per square meter of sea surface. Contour interval 50, 100, 200 cc. per square meter.

The Seasonal Distribution of the Zoöplankton Population

The quantities of the catches obtained by vertical hauls, and their positions during the most complete periods of survey, are entered on the charts shown in Figs. 1 and 2. These charts show that the area of maximum abundance shifts its position with the season. From late summer until December the richest population is found in the northern portion of the Gulf, centering off Mount Desert. During the winter the center shifts to the west, coming to lie off the Massachusetts coast.

In late spring and early summer the richest catches were obtained along the southern margin of the Gulf, north of Georges Bank, extending from the offing of Cape Cod, eastward and northward toward the Bay of Fundy.

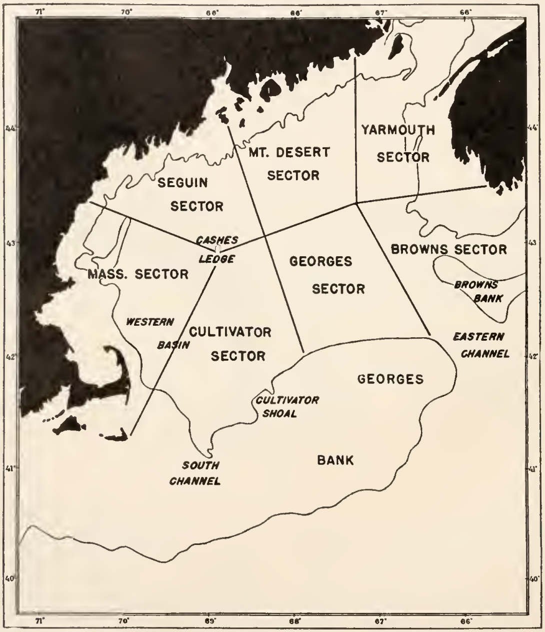
71°
70°
69°
68°
67°
66°
44°
43°
42°
41°
40°
YARMOUTH SECTOR
MT. DESERT SECTOR
SEGUIN SECTOR
CASHES LEDGE
MASS. SECTOR
GEORGES SECTOR
BROWNS SECTOR
BROWNS BANK
WESTERN BASIN
CULTIVATOR SECTOR
EASTERN CHANNEL
GEORGES BANK
CULTIVATOR SHOAL
SOUTH CHANNEL

FIG. 3. Chart of the Gulf of Maine showing principal place names and the sectors into which the area is divided for analysis of population distribution. Contour encloses depths less than 100 meters.

In order to deal with the data statistically, the area of the Gulf has been divided into seven sectors as shown in Fig. 3. Each sector includes one of the principal lines of stations at which collections were regularly made. The quantities of plankton taken at each cruise in each sector have been averaged and the resulting number taken to represent the density of population in that sector at the time. While the data are frequently numerically inadequate, certain interesting regularities appear from its analysis.

Figure 4 shows the density of population in the various sectors at each principal period of survey. It presents graphically the shift in the center of population westward from the Mount Desert to the Massa-

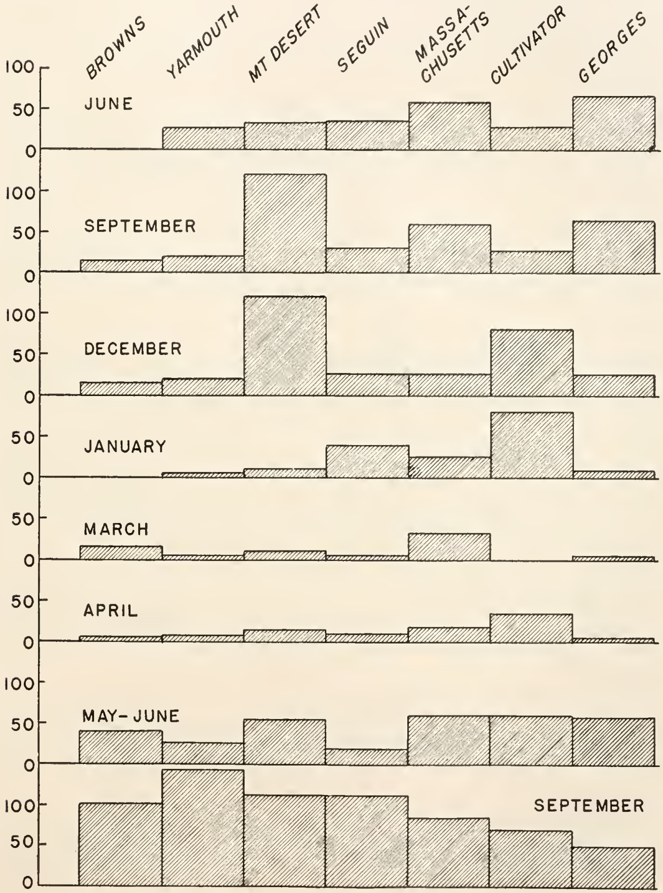

FIG. 4. The average catch in each sector of the Gulf of Maine during the period September, 1933 to September, 1934. Ordinates: cubic centimeters of zoöplankton per square meter of sea surface.

chusetts sector in the course of the winter and its extension along the southern sectors in May and June, followed by the reëstablishment of a maximal population in the northeastern sectors in September.

Figure 5 presents the same data in a form which brings out the sea-

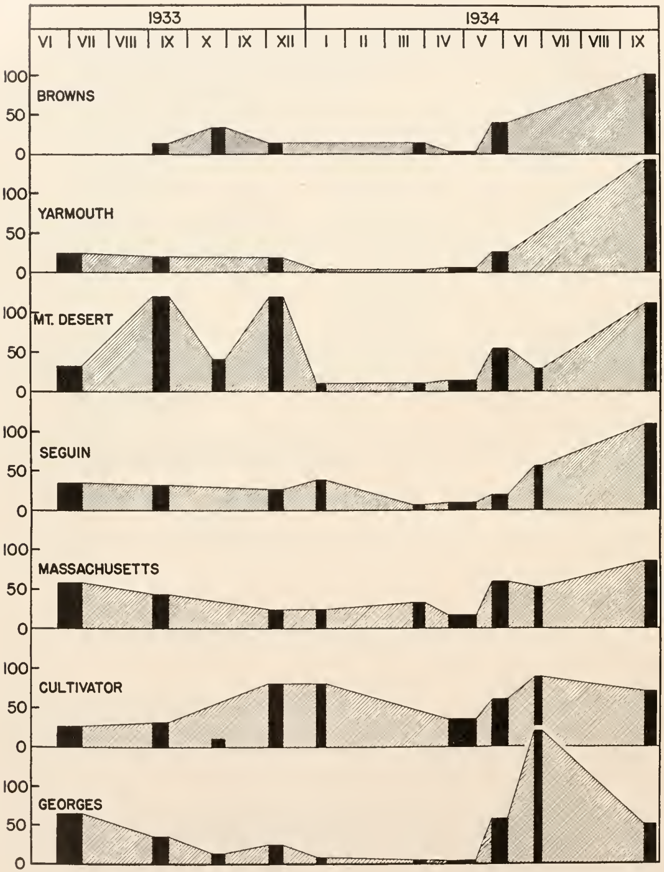

Fig. 5. Seasonal fluctuation of catch in each sector of the Gulf of Maine during the period June, 1933 to September, 1934. Ordinates: cubic centimeters of zoöplankton per square meter of sea surface. The black bars indicate the actual period occupied by each cruise.

sonal fluctuation in each sector. The sectors along the east and north sides of the Gulf are marked by a pronounced seasonal fluctuation—most extreme in the Mount Desert region. In contrast, the population is much more uniformly distributed from month to month in the Massachusetts and Cultivator sectors, which include the greater part of the western basin.

The general features of the distribution appear to recur from year to year, for our observations for September 1933 and 1934 show essentially similar patterns, as do also those for May–June, 1934 when compared with May, 1936. The seasonal distribution observed by Bigelow over a number of years is also in agreement. He found the quantitative fluctuations to be comparatively narrow from season to season in the waters of the western basin and considered the plankton in that part of the Gulf to be " rich " the year round. He reports the northern corner of the eastern basin, as well as the shallows off Cape Sable, to be the site of a wide seasonal fluctuation (Bigelow, 1926, p. 89).

The Circulation of the Gulf

The shift in the center of abundance of the zoöplankton population suggests that it is being borne about a great cyclonic eddy. We may consequently examine the nature of the circulation of the Gulf to see if it can account for the fluctuations in numbers in different places and to learn to what extent the calanoid community may be carried into and out of the Gulf by water movements.

The evidence marshalled by Bigelow (1927)—measurements with current meters, drift-bottles, temperatures, salinities, distribution of plankton, and dynamic calculations—can be harmonized with one type of dominant circulation only, a general anti-clockwise eddy around the basin of the Gulf. The demonstration of this, named by Huntsman (1924) and by Bigelow the " Maine " or " Gulf of Maine " eddy, with all it implies in its biological bearing, is perhaps the most interesting result of their joint explorations of the Gulf. Observations made during a series of years demonstrated that the center of the eddy shifted its precise location from summer to summer, and that marked seasonal variations in the circulatory scheme occurred. Observations of the velocity of the non-tidal drift of the surface made in shoal water about the margin of the Gulf indicated an average movement of seven miles per day, at which rate some three months would be required to complete the circuit of the eddy. No estimations were made of the velocity of the deeper layers.

The hydrographic data collected in the course of the cruises in 1933–34 have been analyzed by Dr. E. E. Watson, who has kindly per-

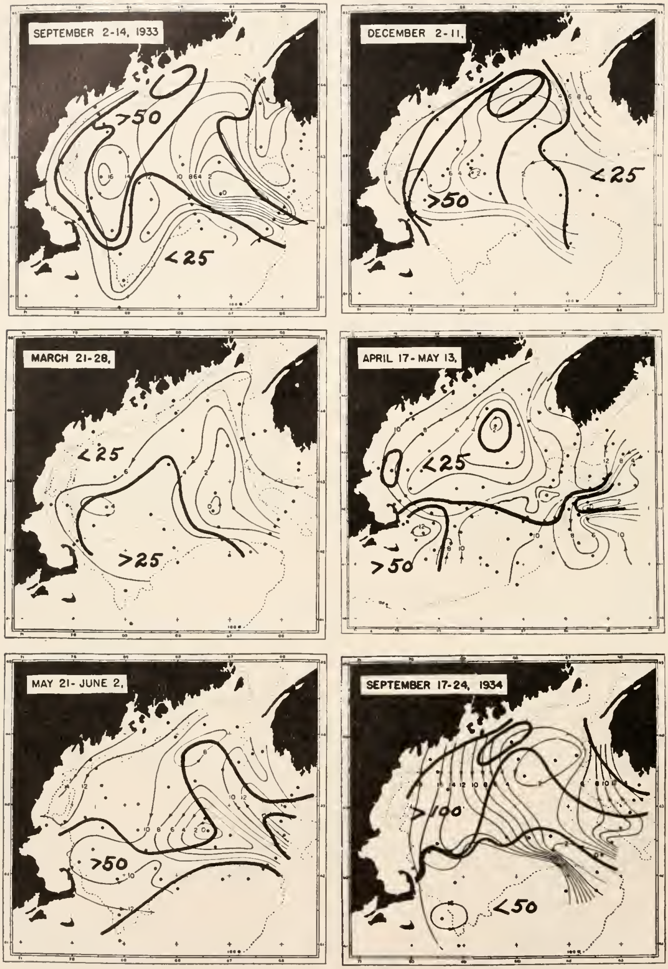
SEPTEMBER 2-14, 1933
>50
<25
DECEMBER 2-11,
<25
>50
MARCH 21-28,
<25
>25
APRIL 17-MAY 13,
<25
>50
MAY 21-JUNE 2,
>50
SEPTEMBER 17-24, 1934
>100
<50

FIG. 6. Dynamic contour charts showing the theoretical circulation of the Gulf of Maine at the surface between September, 1933 and September, 1934. The heavy contours, taken from Figs. 1 and 2, indicate the relative density of population at the time of each cruise.

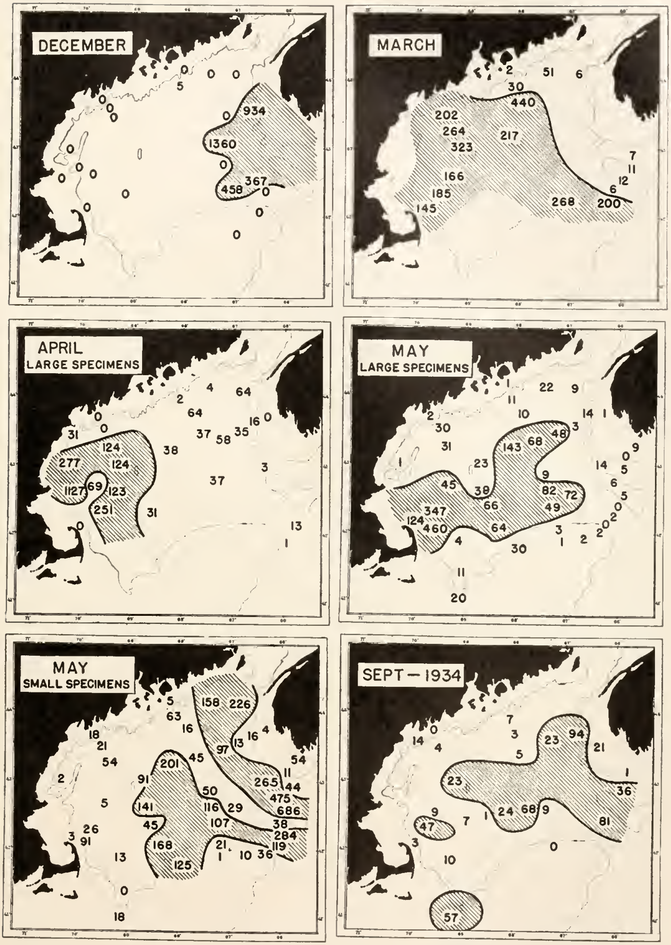
DECEMBER
MARCH
APRIL
LARGE SPECIMENS
MAY
LARGE SPECIMENS
MAY
SMALL SPECIMENS
SEPT — 1934

FIG. 7. The distribution of *Limacina retroversa* in the Gulf of Maine between December, 1933 and September, 1934. The numerals indicate the position and the numbers caught per haul. Compare Fig. 6 for corresponding current diagrams and the coincident distribution of the calanoid community.

mitted me to use some of his current diagrams in advance of the publication of his full report. This study has not only confirmed the more general conclusions of Bigelow, but gives the best available evidence of the actual character of the circulation at the time of our collections.

The zoöplankton population is not distributed uniformly in waters of various depth but tends on the whole to congregate in the upper 100 meters (Bigelow, 1926, p. 93). In many species, particularly of the numerically important copepods, there is a pronounced diurnal vertical migration which has been studied in the Gulf of Maine by Clarke (1933, 1934). At a station in the deep part of the Gulf, he found that *Calanus* and *Metridia* migrated to a depth of 120 meters or more during the daytime and moved upward to levels of from 6 to 42 meters at night. On Georges Bank *Calanus* was confined to the surface strata, undergoing very limited migration, but *Metridia* carried out an extensive vertical migration. Consequently the population cannot be identified exclusively with any particular layer and any attempt to correlate its distribution with the drift of the water is complicated by the undoubted migration of the animals to and from layers of different depth moving with different velocities and in some places without doubt in different directions. As a first approximation, however, it is reasonable to assume that considerable volumes of the more superficial water, unconfined in its movements by shoals, will retain for appreciable periods a unity sufficient to permit a definite population to be identified with it. The horizontal movement of the water at a depth of 40 meters should be fairly representative of the layers in which the zoöplankton chiefly occur within the Gulf. At this depth the water is unobstructed in its movements by any considerabe shoals. We have reproduced the dynamic contours at a depth of 40 meters in a recent paper (Redfield and Beale, 1940, Fig. 10). The surface circulation does not differ in important detail from the charts representing conditions at 40 meters. It shows a somewhat closer correlation with certain features of the plankton distribution. Charts showing the gradient currents at the surface have consequently been employed in preparing Fig. 6.

Additional evidence of the character of the circulation, and particularly of its influence in actually transporting a pelagic population, is provided by the distribution of *Limacina retroversa* in the Gulf during the period of this survey (Redfield, 1939). These organisms appeared en masse in the Browns sector in December and their drift was followed as they spread across the Gulf. In four months they had arrived in numbers in the western basin, having spread along the northern margin of the Gulf in the course taken by the receding center of the zoöplankton population (Fig. 7).

The Relation of Population Distribution to the Hydrography of the Gulf

The following theory is proposed to account for the seasonal fluctuation of the population of zoöplankton. The superficial current, or nontidal drift, consists of a great cyclonic eddy. The eddy is augmented by the inflow of water on the eastern side from over the Nova Scotian Banks. The inflow is compensated for by the escape of water to the south and east across the end of Georges Bank. The relative volumes of inflow and outflow vary from season to season and year to year. In the winter and early spring the inflow is sufficiently great to replace a considerable part of the eddy with water new to the Gulf. This "new" water is relatively barren and does not develop a more considerable population until conditions become favorable for growth and reproduction in the spring, by which time it has extended over the entire northern half of the eddy. Meanwhile an equal part of the older water, which had been in the Gulf during the preceding summer, escapes from the Gulf. The remainder occupies the southern half of the eddy. This water supports a rich population grown up during the previous summer and only moderately diminished by the conditions of the winter. In spring and summer the inflow and outflow diminish and the southern half of the eddy carries an increasing quantity of water northeasterly toward the Bay of Fundy, with the result that this water enters a second circuit of the Gulf, carrying with it a large population which enriches the northern half of the eddy during the late summer and fall. This region may also be enriched by an inflow of water from the Nova Scotia banks which carries a considerable population at some seasons.

The adequacy of this theory is demonstrated in Fig. 6, in which contours representing the areas of relative abundance recorded in Figs. 1 and 2 are transposed upon diagrams of the dynamic gradients in the surface waters. The general distribution of these contours was established without reference to the current diagrams.

In September, 1933, the center of population lay in relatively quiescent water along the northern margin of the Gulf and extended southwestward to occupy a secondary eddy over the western basin. A more scanty population occupied the Cultivator sector. Over the Nova Scotian Banks a condition of slack water existed with no evidence of indraft except along the eastern margin of the Eastern Channel. This water was scantily populated. A marked eddy occupies the Eastern Channel with its offshore component lying on the western side. This includes a tongue of richly populated water in which much plankton is being carried out of the Gulf. During this period the population is being impoverished by this outdraft.

By December a strong indraft of water over the Nova Scotian Banks has commenced carrying into the Gulf water containing a scanty population. This water contains abundant *Limacina* which occupy the eastern area in which catches of less than 25 cc. of plankton occurred. (Figure 7.) Compensatory movements must be expelling richly populated waters over Georges Bank.

By March scantily populated water has extended along the entire northern margin of the Gulf, carrying with it the population of *Limacina*. Considerable volumes of zoöplankton were then taken only in the southwestern quarter where relatively slack water is found. A small eddy occupies the western basin and in it *Limacina* mingle with considerable remnants of the copepod population. In the eastern half of the Gulf the major eddy is well marked. Along its eastern side water still enters the Gulf scantily populated with copepods and now containing very few *Limacina*. Its western arm is carrying many *Limacina* out to sea.

In April the character of the circulation changes abruptly from a loop to a closed eddy. Invasion of water from offshore has come practically to an end and considerable numbers of copepods which occur at the mouth of the eastern channel have no opportunity of entering the Gulf. In the west a small concentration of copepods persists near the South Channel. The *Limacina* population is now centered over the western basin, but considerable numbers appear to have spread eastward along the southern and eastern arcs of the great eddy.

Up to this time the movements of water and of the populations of copepods and *Limacina* appear to be perfectly correlated. There can be no doubt that the inflow of barren water from the east has displaced a large part of the copepod population from the northern and eastern part of the Gulf, forcing it out to sea over the eastern end of Georges Bank.

In May and June the loop-like character of the circulation reëstablishes itself, but a considerable eddy persists in the center of the loop. Reproduction now increases the population everywhere. The copepod population occurs in greatest numbers in the slack water of the western basin. From there a rich band extends along an east-flowing current out to sea over the Eastern Channel. A part of this eastward extension has evidently been caught in the recurrent eddy and carried northward toward Mount Desert. Richly populated water found in the North Channel appears to be moving out of the Gulf. The only water entering the Gulf at this time lies along the east side of the Eastern Channel and appears to be scantily populated. It seems improbable that a considerable population is being recruited at this time. The increasing numbers observed in the eastern region apparently come from the southern and western region.

The distribution of *Limacina* in May–June agrees with this interpretation of the water movements. The population of large specimens, which had wintered in the Gulf, extends eastward along the southern side of the Gulf, and northward along the eastern side of the eddy. The small specimens, new to the Gulf, lie along the inflow and about the center of the eddy. Others follow the eastern arm of the inflow which recurves along the Nova Scotia shore.

By September, 1934 conditions have reëstablished themselves much as they were a year before. The southern half of the Gulf appears to be occupied by the more scanty population, presumably derived from the barren water which lay to the north in the spring. This is trapped in a dead water. A large population has grown up in the eddy which forms each summer in the northeast quarter and well-marked currents exist to carry this population to the southeast. The current flow into the Gulf is stronger than the year before and appears to bear an abundant population from offshore into the eastern side of the Gulf. This is the only indication that the copepod population is enriched by exchanges with offshore waters in the course of the year.

The small numbers of *Limacina* which occupied the Gulf in September, 1934 occurred in greatest numbers along the course of the inflowing water.

It is unfortunate that a more complete survey was not made between June and September. The events which are least clear are those leading to the development of the exceptional populations in the Mount Desert region in late summer. It is not certain that these may not have been recruited from offshore during the summer. The circulation calculated for May–June would appear to transport the richer water then found in the south out to sea more effectively than toward the northeast. Possibly the rich population extending northward toward Mount Desert arrived there before the loop-like eddy reestablished itself. There are, however, several considerations which support the view that the population of the northeastern sectors is recruited from the southern part of the Gulf in early summer.

Fish (1936) records the invasion of the coastal waters of Maine by *Calanus finmarchicus* larvae in June which he assigns to a " western stock." These he believes to be absent from the eastern half of the outer Gulf earlier in the season, and to have drifted in toward the Maine coast from the southwest. The larvae of this stock greatly outnumber those of an " eastern stock " which entered the Gulf from the Scotian Banks in April.

Drift-bottle observations indicate an actual movement of water from the southern toward the northeastern quarter of the Gulf in summer (Bigelow, 1927). There can be no doubt that the surface water does move in this direction, dynamic calculations notwithstanding.

It is possible that lateral mixing along surfaces of equal density may permit rather extensive exchanges of water across gradient currents (Iselin, 1939). In particular, according to a principle developed by Parr (1936), stratification in turbulent waters leads to increased lateral mixing. Thermal stratification in the Gulf of Maine was well developed in May, 1934, and its onset may have facilitated the transfer of well-populated waters across the eddy. It is noteworthy that during May a considerable intermingling of *Limacina* with water rich in copepods occurred along the southern side of the Gulf. It is also noteworthy that the distribution of *Limacina* became much more homogeneous in May than it had been earlier. This was true also of the plankton population as a whole, as Fig. 4 shows. Lateral mixing deserves more study by biologists, as Iselin has pointed out, for it may well be an important factor in preventing local breeding stocks from being swept out of embayments by directional currents.

In summary, the hydrographical evidence appears to support the view that the scanty population of the eastern sectors in midwinter is due to their occupancy by the barren water, which appears in the Browns Bank sector in December and can be traced until it enters the Massachusetts sector by May. The eastern and northern sectors thus receive an influx of relatively barren water in midwinter when the climate is unfavorable for further growth of the population. In the early summer, on the other hand, water drifts from the southern part of the Gulf into the northeastern sectors. This water is about to commence its second circuit of the Gulf and carries with it a population which has already grown to some magnitude in the sectors from Massachusetts to Georges Bank by the end of May.

In contrast to this, the sectors of the western basin receive in winter water which had acquired an abundant population in the Mount Desert and Seguin sectors during the fall. Although there is some destruction of the organisms at this season, it is not sufficient to reduce the numbers greatly. With the coming of spring, this water moves on to be replaced by the barren water found in the northern sectors during the winter. But as this water warms, its population grows and rapidly comes to equal that of the water it replaces. The uniformity of the population in the western basin is due to the fact that a rich fauna arrives there coincident with unfavorable conditions in winter, and a scanty fauna comes to occupy the region during the period most favorable for growth.

The Average Monthly Catch

The monthly catch obtained by averaging the mean values for all sectors during each cruise is given in Fig. 8. The average monthly catch remained constant at about 40 cc. per square meter from June to December, 1933. The values fall markedly from January through April. This is undoubtedly due to the destruction of the population by winter cold. At this period, however, large quantities of water poor in population are entering the Gulf and an equivalent quantity bearing a richer population is leaving, thus accounting for much of the loss.

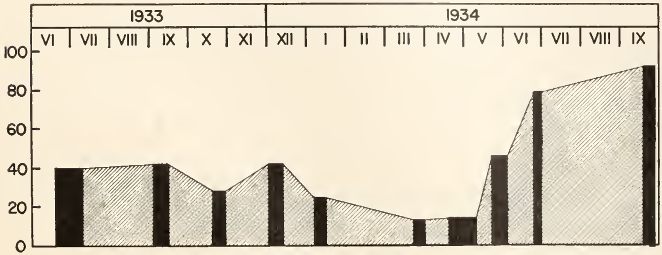

Fig. 8. Average catch for entire Gulf during the period of survey. Ordinate: cubic centimeters of zoöplankton per square meter of sea surface. The black bars indicate the actual period occupied by each cruise.

In May the average population begins to increase suddenly and its growth continues at a diminishing rate until the last observations in September, 1934. The population as a whole is then twice as great as that encountered during the preceding year,—the average haul being 90 cc. per square meter.[2]

[2] Bigelow found that the zoöplankton was at its lowest ebb in late February and the first half of March in 1920. At this time his catches varied from 75 to 25 cc. per square meter measured wet (equivalent to 15 to 5 cc. measured "dry"). Our catches in March averaged 13 cc. and for April 8 cc. per square meter measured "dry." The 1934 year appeared to be a late year and was initiated by an unusually cold winter. The April observations are perhaps misleading since collections were not made in the parts of the Georges and Cultivator sectors where the highest population was expected. Bigelow considered 100 cc. wet (20 cc. or more measured dry) to be representative of the Gulf in midsummer, a value much smaller than our average of 40 cc. in 1933 and 80 cc. in 1934. His largest catch of 425 cc. (85 cc. "dry"), made in September, 1915, does not exceed the average value obtained in September, 1934 for all sectors. It must be remembered that he employed a different method of measuring his catch and nets which doubtless differed from ours in efficiency.

The Total Annual Production and Exchange

The average catch throughout the period of the survey in each sector,—obtained by averaging the mean figures obtained at the time of each cruise—is given in Fig. 9.[3] It is noteworthy that the catch increases progressively as one passes along the course of the water movement from its inflow over the Browns Bank sector to its exit across the end of Georges Bank. The longer the water has been in the Gulf, the greater its population.

The average catch is greatest in the Mount Desert sector and is an exception to the foregoing tendency. We suggest that this is due to the movement of water, which has already completed the circuit of the Gulf and is rich in plankton, from the southern sectors into the Mount Desert sector during the summer. The Mount Desert sector thus virtually occupies a position at the end of the series during the months when its population is greatest.

The average haul for all sectors of the Gulf and at all cruises is about 40 cc. per square meter of " dry " plankton. If the area of the Gulf be taken at 36,000 square miles, this would indicate a total population of about 3.7×10^{12} cc. or some four million tons.[4]

The standing crop does not give a measure of the rate of production of the population, since it reflects merely a balance between rate of growth and death and the gains and losses in the population by movement into or out of the region. It is clear from Fig. 8 that the crop increased by nearly 80 cc. per square meter of surface between May and September. This represents a net gain of some eight million tons.

The observations make it apparent that the Gulf loses at least one half of its population through the escape of water over Georges Bank and the Eastern Channel each winter. There is no evidence that water enters the Gulf at any time carrying a richer population than that obtaining there at the time, and only in September, 1934 was water found to enter the Gulf in which the copepod population was not distinctly scanty. Unless further observations during the summer should prove the contrary, it may be concluded that the Gulf is a region of production for the calanoid community which supplies immigrants to the southern banks in quantity, but receives relatively unimportant recruitments from the regions to the eastward.

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[3] This method of averaging avoids overweighting the yield of these sectors in which an unusual number of rich hauls were made at a time when the population was particularly large, as would be the case if all catches were simply averaged.

[4] In discussing the productivity of the Gulf of Maine, Bigelow concluded that the population was greatest over a band extending from the Massachusetts coast to Penobscot Bay and the Bay of Fundy. The areas occupied approximately by our Seguin sector and the greater part of the Cultivator and Georges Bank sectors were considered barren. His conclusion that the southern sectors are barren certainly rests on insufficient evidence, since only two hauls are recorded. Our richest haul was obtained over the southeastern deep in June, 1934 and the Cultivator and Georges sectors are the most populous sectors excepting Mount Desert throughout the year as indicated. Our observations agree that the Seguin sector is relatively unproductive. Its best season was the fall of the year, a time when Bigelow made relatively few cruises. Several of our richest hauls were made off Seguin in September, 1934.

The Growth of the Population in the Moving Mass of Water

If the interpretation which we put on the data is correct, it is certain that observations made at a geographically fixed point, or standard station, tell little about the fluctuations of any unit of the population. We record simply a series of events distributed in space as they drift past in the course of time. The conditions we observe today are not determined by the events we observed yesterday. The curves of population growth presented in Fig. 5 are grossly misleading if they are interpreted to represent the history of any biologically continuous unit.

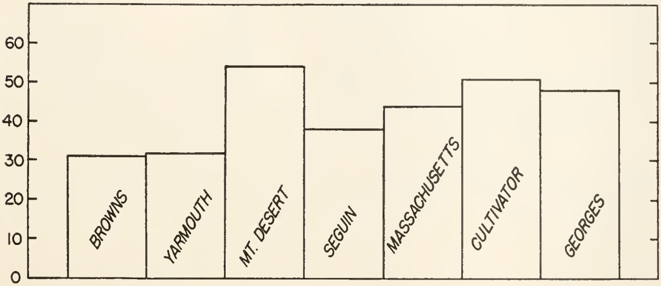

Fig. 9. The average catch in each sector during the period June, 1933 to September, 1934. Ordinate: cubic centimeters of zoöplankton per square meter sea surface.

By taking account of the rate of drift of the water, it is possible to select appropriate stations to show the growth of population in a unit volume of water as it is carried about the Gulf of Maine eddy at the apparent rate of its non-tidal drift. While the result is both an abstraction and an approximation, it probably indicates the true history of events better than the usual curves of population fluctuation. Figure 10 represents the apparent growth of the population of a unit mass of water entering the Gulf in December and recognized by the presence of *Limacina*. As the season advances it is carried across the northern sectors, arriving by May in the offing of Cape Ann. During this period the population decreases by about one-third, but in May as it crosses the western basin rapid growth occurs with the result that in midsummer it has reached the value of 50 cc. per square meter, greater than the yearly average for the Gulf as a whole. The unit may now drift out of the Gulf across the end of Georges Bank in late summer or it may drift northeasterly into the Yarmouth sector to commence a second circuit of the Gulf. Its numbers grow meanwhile to over 100 cc. per square meter. After January 1, high mortality again reduces the numbers to about one-third or to 30 cc. per square meter, as it crosses the Massachusetts sector in March. In May growth recommences and by midsummer it reaches the Georges sector, having attained the record volume of 170 cc. per square meter. Its history may be terminated by supposing it to be carried out of the Gulf at this time.

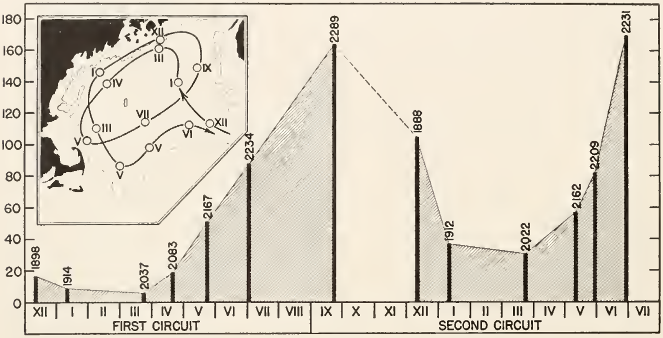

Fig. 10. The growth of population in a mass of water assumed to move along course indicated in inset. Ordinate: volume of zoöplankton in cubic centimeters per square meter sea surface; abscissa, time in months. The black bars indicate the volumes caught at the selected stations. The positions of these stations and the month of collection are indicated on the inserted chart.

Figure 10 is presented because it illustrates the possibility of taking account of the current system in an ecological analysis. It may be pointed out that the life history of any individual species might be treated in a similar way.

Annual Fluctuations in Population

The average catch at all stations was more than twice as great during the summer of 1934 as during the same period in 1933. Since the hauls were numerous, widely spaced, and all made with the same technique, there can be little doubt of the significance of this observation. The difference is the more striking in that an unusually severe winter preceded the richer year.

The suggested theory offers a tentative explanation of such fluctuations. The poverty of the water in the northern half of the Gulf in winter seems due to the introduction of a large volume of relatively barren water from the Scotian Banks. It is suggested that more of this water enters in some years than in others, and that the population of the Gulf is impoverished in proportion to the magnitude of this inflow. The longer water remains in the Gulf, before being replaced by new water, the richer its population becomes.

A number of facts support this suggestion. It is well known that the magnitude of the inflow varies from year to year. The inflow during the winter of 1934 seems to have been of shorter duration than usual, having been completely terminated by a strong movement of water from the Gulf over the North Channel and adjoining banks in May. The movement was apparently underway in the latter part of April, as the current diagrams show (Fig. 6). Drift bottles set out by Dr. Herrington off Cape Sable at that time were recovered to the eastward. In contrast to this, Bigelow observed the invasion of the Gulf by Nova Scotian water to continue until May or June and to result in a cooling of the eastern part of the Gulf long after the other parts had commenced to warm. He speaks of the invasion as a phenomenon of spring, whereas in 1934 it terminated before the end of winter.

It is also possible that differences in the circulation such as those observed in September, 1933 and 1934 may cause the population of the Gulf to be augmented from external sources to a different degree each season.

Since the water flowing into the Gulf over the Nova Scotian Banks is less saline than that occurring at like depth within the basins, a small influx of this water should be followed by a summer of relatively high salinity. The superficial water of the Gulf was exceptionally salt in 1934. It appears to have been as salt or salter than in 1915, the most saline year recorded by Bigelow. In 1933, on the other hand, the water was quite as fresh as in 1914 and 1916, the least saline of those he recorded. A correlation between salinity, productivity, and annual inflow over the Scotian Banks is strongly suggested. Systematic annual observations would serve to test this relation, and should it prove general, might lead to an understanding of the yearly fluctuations in commercial fisheries.

The Distribution of Petrels and Mackerel

The zoöplankton supplies food for various predators. Their distribution may be expected to be influenced secondarily by the hydrographic factors which determine the abundance of the calanoid community.

Petrels

These birds appear to feed upon zoöplankton or their products. They pick up whatever scraps of organic matter they can find, gathering about fishing vessels, following ships, and feeding about the carcasses of dead whales and seals. There is a general belief that they pick up droplets of oil from the surface of the water, and their stomachs frequently contain an oily fluid which they eject when captured. It seems more probable that this is derived directly from their food. Wilson (1907) states that the food of the Wilson's petrel, which he observed in the antarctic, consists of minute crustaceans. The natural food of the Leach's petrel, according to Bent (1922, p. 143) " includes shrimps and other small crustaceans, floating mollusks, perhaps small fishes occa-

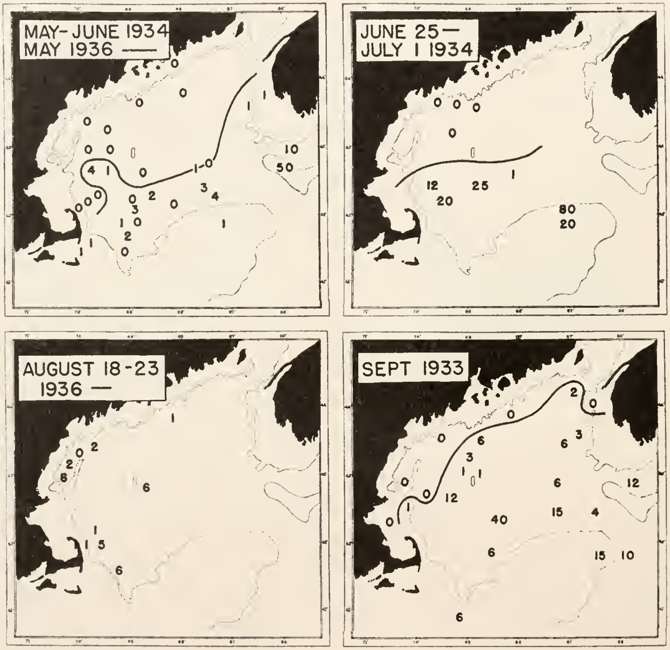

FIG. 11. Numbers of petrels observed in different parts of the Gulf of Maine during cruises at various times of year.

sionally, and probably many other forms of minute marine animals which are found swimming on the surface or in floating masses of seaweed."

Wilson's petrel is the common petrel of the Gulf of Maine. Leach's petrel, though a breeder along the coast of Maine, is much scarcer. Not more than one petrel in twenty or thirty observed at sea is of this species. Wilson's petrel breeds in the south Atlantic during December, January and February and does not reach the Gulf of Maine until May. None were observed during the cruise of April 19–23, 1934. In cruises in May, 1934 and 1936 petrels were observed in small numbers in the southern and eastern regions, where at that season the largest zoöplankton hauls were taken, but were absent from the northern sectors which at the time were occupied by relatively barren water (Fig. 11). During a cruise from June 25 to July 1, 1934, petrels were present in larger numbers in the southwestern quarter, but were not observed in the Seguin sector. On August 18 to 23, 1936, however, petrels were observed regularly at stations made in the northern side of the Gulf. In September, 1933 petrels were observed everywhere in the Gulf except at a few coastwise stations along the shore. At this time all sectors supported an abundant population of zoöplankton.

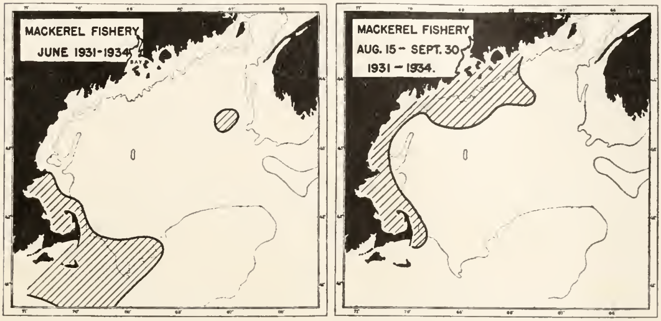

Fig. 12. The areas occupied by the mackerel fishery during the early and late summer 1931–1934.

It appears that when Wilson's petrel first arrives in early summer it remains confined to those sectors of the Gulf which then support the richest plankton.

Mackerel

In the Gulf of Maine these fish have long been known to feed on calanoid copepods and are known to eat various other crustaceans which compose the bulk of the zoöplankton (Bigelow, 1926, p. 102). It is not surprising in consequence to find a correlation between the seasonal distribution of this fishery and of the local abundance of zoöplankton.

Dr. Settee has kindly placed at my disposal the very complete records made by the U. S. Bureau of Fisheries showing the places in which mackerel have been taken during recent years. The mackerel fishery begins in New England waters with the arrival of the fish south of Cape Cod in May and June. The fish are first taken along the shores of the Gulf of Maine in June, chiefly within the 100-meter contour from Cape Ann to Cultivator shoal. At this time heavy catches are also made along the east coast of Nova Scotia and northward to Gaspé (Settee and Needler, 1934). The July fishery has much the same distribution, though tending to spread farther east along Georges Bank and also in some years along the western part of the coast of Maine. In August and September a considerable fishery is conducted in the northern side of the Gulf, from Mount Desert westward, and southward as far as Cape Cod (Fig. 12). During these months the fishery in the Bay of Fundy is at its height, 37 per cent of the total catch being made during each of these two months, whereas less than 10 per cent is taken in any one of the preceding or following months.

In interpreting these facts the peculiarities of the fishermen as well as of the fish must be borne in mind. Mackerel are now marketed fresh and are landed chiefly in Boston. The fishermen consequently do not fare farther from this port than is necessary. The takings of mackerel do not reflect accurately the total distribution of the fish, but only their availability to the Boston market. It seems sufficiently clear that in early summer mackerel are available chiefly along the southern shores of the Gulf; that by late summer their abundance has shifted to the northern shores, including the Bay of Fundy. This is the distribution of the maximum of zoöplankton population as well. Whether the mackerel follow the plankton as it drifts around the great eddy, or cut across to meet its advance from the east in the late summer, as the fishermen undoubtedly do, cannot be told.

Thus there appears to be a general correlation between the distribution of the zoöplankton, the occurrence of petrels, and the capture of mackerel.

SUMMARY

1. The seasonal and geographical fluctuations of the abundance of the calanoid community of the Gulf of Maine are described.

2. The shift in the center of abundance is closely correlated with the superficial circulation, deduced from hydrographic observations and the drift of an invading population of *Limacina*.

3. The principal factor influencing the distribution of population density is the inflow of relatively barren water from the Nova Scotian coast in winter.

4. The Gulf appears to be an area from which the calanoid community spreads to other waters, but which receives relatively small recruitments from without its borders.

5. A breeding stock is maintained by the establishment of a recurrent eddy in the late spring.

6. Estimates of annual productivity and seasonal mortality are given.

7. The distribution of petrels and of the mackerel fishery appears to be correlated with the distribution of zoöplankton.

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CHANGES IN THE TISSUE CHLORIDE OF THE CALIFORNIA MUSSEL IN RESPONSE TO HETEROSMOTIC ENVIRONMENTS[1]

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The adult California mussel, *Mytilus californianus* Conrad is a fairly heterosmotic animal, typically marine in its environment, yet potentially euryhaline in a striking degree.

It has been shown (Fox et al., 1936) that this mollusk can live in the laboratory for long periods following sudden and continued immersion in aerated solutions of natural sea salts, varying in Cl concentration[2] from about 0.94 per cent, or approximately half that of natural sea water in the vicinity of La Jolla, to as high as 2.5 per cent, or about 34 per cent above normal values (1.86 per cent being the approximate Cl concentration of sea water at La Jolla).

Sudden exposure to concentrations of sea salts below or above these respective values proved fatal to mussels, but gradual alteration of the water in which they were immersed, over periods of several weeks, left the animals still alive in solutions diluted, on the one hand, to one-third of the normal Cl concentration (0.62 per cent Cl) or concentrated, on the other hand, to nearly twice the normal value (3.50 per cent).

The purpose of this investigation was to determine the chloride concentration in the tissues of the mussel, under the widely differing physiological conditions which must result from continued exposure to concentrations of sea salts not encountered by these animals in nature.

Extensive reviews of both older and more recent work dealing with homoiosmoticity and heterosmoticity of fishes and invertebrates have been presented in papers by Dakin (1935) and by Schlieper (1935).

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[1] Contributions from the Scripps Institution of Oceanography New Series No. 117.

[2] Cl concentration refers to the total halide ion concentration as determined in sea water analyses. The same designation, used in the present discussion, refers to the total halide concentration in parts per cent of both water and tissues, since the amounts of both Br and I are relatively very small. Concentration of "chloride" refers hereinafter to that of halide ions.

Other useful discussions of the subject are given by Adolph (1930) and by Baldwin (1937).

Experimental Methods

Diluted sea water solutions were prepared by adding distilled water to sea water, while relatively concentrated solutions were obtained by the partial evaporation of ordinary sea water without precipitating any salts.

Experimental solutions were contained in glass battery jars, each of 2½ gallon capacity. To avoid any injurious effects which might result from overcrowding, the predetermined ratio of at least one liter of water per animal was consistently adopted as a minimum. Mussels of an average length of 10 cm. (varying between 9 and 11 cm.) were employed in the great majority of experiments, although no biochemical or physiological differences of direct bearing upon the work were recognized in larger animals (12 cm.) or in somewhat smaller ones (7 to 8 cm.)

Experiments were carried out at room temperatures which varied between the approximate limits of 19° and 22° C. All solutions containing mussels were aerated continuously.

No attempts were made to adjust and maintain the pH of the various solutions, since it was found that mussels lived in piped sea water within the range of pH values encountered in the experiments. Figures representing numerous pH measurements of environmental solutions will serve as examples and are tabulated below. These measurements were made with a Beckman glass-electrode pH meter through the kindness of Mr. J. C. Hindman.

Solution	pH
(1) Sea water of normal salinity from running supply in tank containing mussels; unaerated	8.63
(2) The same; aerated	8.43
(3) The same, drained from mantle-cavity of mussel; unaerated	7.51
(4) Sea water of normal salinity from stationary supply in large jar containing mussels; unaerated	7.50
(5) Sea water, initially of normal salinity, diluted to maximum degree used in experiments, i.e. to one-third of original concentration (ca 0.62 per cent Cl); aerated	8.52
(6) Sea water, initially of normal salinity, concentrated to maximum degree used in experiments, i.e. to 54 per cent of original volume (ca 3.50 per cent Cl); aerated	8.32

Since the mussels were therefore presumed not to have been exposed to unfavorable conditions of acidity or alkalinity during the course of the experiments, it was concluded that extensive changes in the concentration of dissolved salts themselves constituted the limiting factors to life in the environment. The possibility of disturbances in " salt balance " (in the physiological sense), of experimental solutions was considered. Because calcium was believed to be the chief element prone to be precipitated as the carbonate from moderately concentrated sea water, analyses were made for dissolved calcium in sea water samples concentrated by boiling. Normally present in amounts close to 0.42 gram per liter, the dissolved calcium in a solution boiled down without precipitation to 54 per cent of its original volume (number 6 in above table) was present in the nearly theoretical amount of 0.80 gram per liter. Since this solution was the most concentrated used in any of the experiments, it was concluded that mussels were at no time exposed to solutions " unbalanced " with reference to calcium.

Experimental animals were introduced into the various solutions of altered salinity after first propping the valves apart by a few millimeters with smooth glass plugs and draining the gill chamber of sea water. Animals immersed under these conditions in sea-salt solutions varying between the approximate limits of 0.95 per cent and 2.73 per cent Cl usually relaxed their hold on the glass rod in a short time, parted their valves in a normal manner, and resumed feeding activities.

The flesh of control and experimental mussels was prepared for chloride analysis in the following way. The mussels were removed from their shells as rapidly as possible and with minimum cutting of tissues, severing only the adductors and small muscles attached to the hinge region of the shells. The flesh was blotted on absorbent paper to remove most of the adhering sea water, then rinsed briefly in 95 per cent ethyl alcohol (and reblotted) in order to remove most of the remaining sea water from body surfaces, constrict the gill capillaries, wash out sea water expressed therefrom, and coagulate the cut surfaces to allay excessive bleeding and subsequent losses of chloride-containing body fluids. The consistent adoption of this procedure resulted in a series of checks which were quite close in normal control animals, in spite of the fact that the work was done on the wet weight basis.

For chloride analysis, the method of Sunderman and Williams (1931, 1933) was followed, digesting the whole tissues in chloride-free KOH, followed by further treatment of aliquot portions with concentrated HNO_3 in the presence of an excess of dilute $AgNO_3$ solution. The excess of Ag ion was finally titrated with standard NaCNS solution in

TABLE I

Chloride concentrations in wet tissues of mussels, showing slight variations with sex, maturity and season.

Date	No.	Sex	Weight of flesh	Percentage Cl		
			grams			
8–11–37	1	♂ (much sperm)	22.88	0.99	av. % Cl in whole ripe ♂ = 0.91 av. % Cl in whole ripe ♀ = 0.87 Grand av. = 0.90	Summer.
8–13–37	2	♂ " "	18.70	0.83		
8–13–37	3	♀ (many eggs)	14.85	0.89		
"	4	♂ (much sperm)	16.57	0.89		
"	5	♀ (many eggs)	16.50	0.82		
"	6	♀ " "	13.23	0.805		
"	7	♂ (much sperm)	17.59	0.83		
8–17–37	8	♂ (mature)	12.51	1.00		
"	9	♂ "	10.025	0.965		
"	10	♀ "	8.52	1.065		
"	11	♂ "	11.50	0.81		
"	12	♀ "	10.61	0.77		
"	13	♂ (immature)	6.56	0.99		
	Gonads only	♂	16.95	0.90	av. % Cl in ripe ♂ gonad = 0.89 av. % Cl in ripe ♀ gonad = 0.57	
	" "	♀	15.68	0.61		
	" "	♂	15.03	0.945		
	" "	♀	13.86	0.58		
	" "	♂	10.24	0.82		
	" "	♀	13.61	0.52		
12–13–37	1	? (immature)	6.41	0.97	av. % Cl in immature animals = 0.98	Winter.
"	2	♂ "	6.26	0.98		
"	3	? "	7.98	0.96		
"	4	? "	8.19	0.98		
"	5	♂ "	5.96	1.005		
"	6	♂ "	5.08	0.995		
1–31–38	1	♀ (many eggs)	12.41	1.00	av. % Cl of sperm—cont'g ♂ = 0.97 av. % Cl of egg—cont'g ♀ = 0.93 av. % Cl of all ♀ = 0.945 av. % Cl of all animals = 0.96	
"	2	♀ (spawned)	9.84	0.91		
"	3	♂ (much sperm)	16.37	0.96		
"	4	♂ " "	18.16	0.97		
"	5	♀ (many eggs)	12.08	0.93		
"	6	♂ (much sperm)	15.69	1.01		
"	7	♀ (many eggs)	17.94	0.905		
"	8	♂ (much sperm)	16.49	0.945		
"	9	♀ (many eggs)	21.20	0.90		
"	10	♀ (spawned)	12.38	1.03		

the presence of ferric alum according to the well-known method of Volhard. The Volhard method was also employed for the analysis of chloride in sea-water solutions. Our experiments showed that the step involving preliminary digestion of tissues by KOH gave consistently higher values for chloride than did the ordinary " open Carius " determinations (digestion with excess concentrated HNO_3 and $AgNO_3$) employed by other workers. Sunderman and Williams (1933) report incomplete recovery of chloride when the preliminary alkaline digestion is omitted, and assign the low chloride values to interference by fatty substances.

Preliminary Analytical Survey of Normal Animals

Because parts of this research were conducted in different seasons of the year, i.e. especially in the summer and fall of 1937 and the winter of 1937–1938, it seemed desirable to compare chloride analyses of normal animals taken during August with those of animals comparable in size and weight taken in December and January. Also, because it was impossible to differentiate the sexes without sacrificing the animals' lives, and since it was conceivable that biochemical differences in sex might be reflected in the chloride content of the tissues, attention was given to the sex and relative degree of maturity throughout the same group of animals.

The data of Table I reveal that differences between the chloride content of whole bodies of summer animals and those of winter animals are of relatively small order, showing a departure of only + 0.06 per cent in the grand average, in favor of the winter animals; this difference is the same in direction and extent whether one compares ripe winter with ripe summer males, or ripe winter with ripe summer females.

Sexually immature animals exceeded in chloride content the grand average (0.93 per cent) of the combined values of mature animals of both seasons by the small departure of 0.05 per cent.

Because the demonstrated seasonal and sexual differences in chloride content fell well within the departures recorded between individual analyses, they were not regarded as significant in the experimental results. The sexual difference in chloride content appeared to be a real one, although relatively small, and for the purposes of this work, insignificant. It was doubtless due to the fact that the relatively heavy ripe ovary contained only about two-thirds as much chloride as did the male gonad.

Changes in Tissue Chloride Following Sudden Immersion in Heterosmotic Solutions of Sea Salts within the Tolerated Physiological Range of Concentrations

Reference to Figs. 1 and 2 brings out some rather consistent general facts: While mussels in nature show close agreement among one another in chloride content, wide individual variations are manifest in the rate of change in tissue chloride concentrations in both kinds of new environment. This is particularly striking in the early hours following immersion. Since mussels have been demonstrated to survive for long periods at either the hypotonic or the hypertonic concentrations here

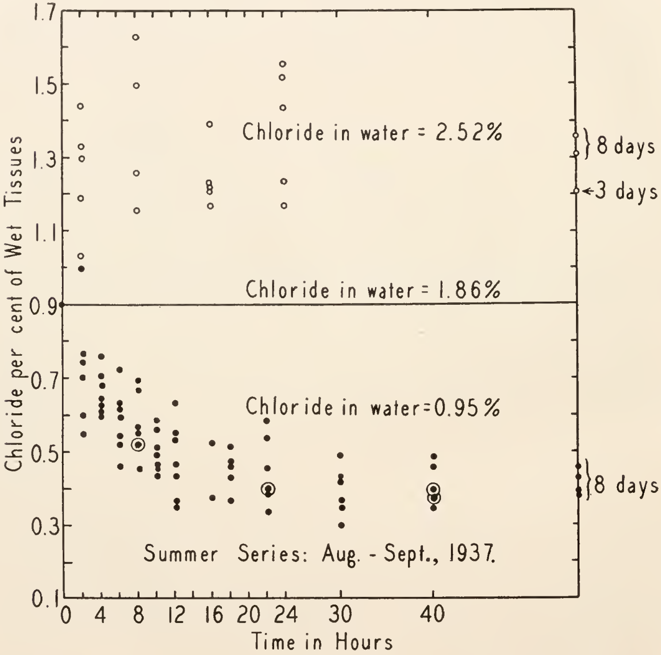

FIG. 1. Chloride analyses of whole mussel tissues following immersion of living animals for increasing time-intervals in hypotonic and hypertonic sea water within the limits of the tolerated range. Each point represents the analysis of a single animal; where two or more analytical values were identical, this is shown by concentric circles.

employed, and to eventually attain, in each group, respective chloride levels in close individual agreement, the obvious differences in the early hours are clearly due to individual variations in rate of water and salt interchange.

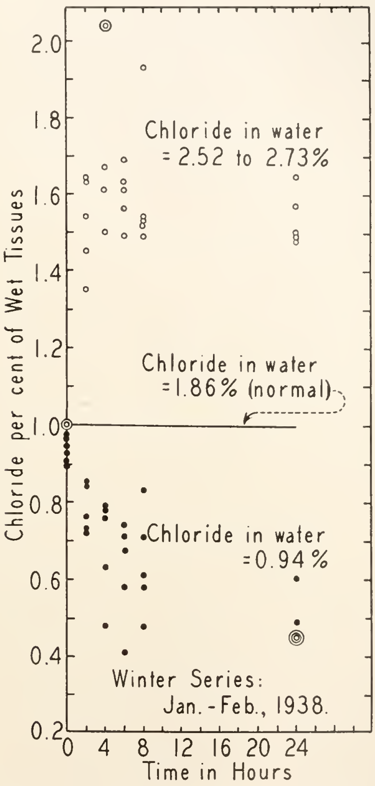

FIG. 2. Further chloride analyses, as in Fig. 1. See Fig. 1 for meaning of points and concentric circles.

While four out of five animals which remained for the whole 24-hour period in water containing 2.52 to 2.73 per cent Cl (Fig. 2) were filtering water, feeding, and voiding feces and thus appeared normal, none of the animals in the water containing 2.78 per cent Cl (Fig. 3) appeared normal even after 30 hours; they maintained a grip on the inserted glass props, failed to filter water, and gave off much mucus, thus giving several signs of physiological disturbance. A later lot, however, exposed to a solution of 2.80 per cent Cl (Table IV) did not show any effects of injury after 25 hours. These facts are taken as evidence that Cl values between 2.70 per cent and 2.80 per cent are close to the threshold of hypertonicity at which the mussels can withstand sudden immersion.

Figure 4, to which reference will be made below, reveals the close fit to a straight line *between water chlorinities tolerated by mussels after sudden immersion.* Attention is directed, for the moment, only to the portion of the graph lying between the ordinate values of 0.95 per cent Cl and 2.8 per cent Cl, illustrating the data shown in Figs. 1 and 2, and those of Table IV, when all average values of internal chloride concentrations attained by mussels in 24 to 40 hours are plotted against chloride concentrations of external water. The ratio between grams Cl per 100 grams wet mussel tissue and grams Cl per 100 ml. sea salt solution has an average value of 1 : 1.89.

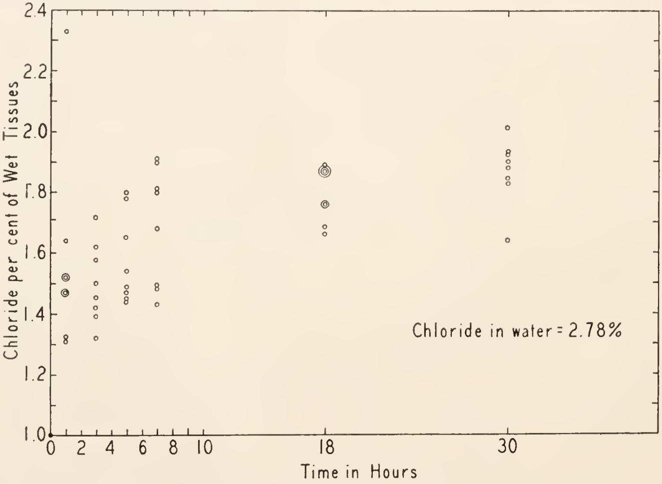

FIG. 3. Further chloride analyses, as in Figs. 1 and 2. See Fig. 1 for meaning of points and concentric circles.

The blackened point on Fig. 4 indicating the average chloride concentration in the tissues of 8 mussels maintained for 30 hours in water containing 2.78 per cent Cl was obtained from Fig. 3. While this average value lies in the vicinity of the curve, its departure is doubtless due to the fact that the animals were in poor condition (from an unknown cause), and so failed to maintain the ratio between internal and external chloride exhibited by other lots of animals at this concentration.

Specimens placed, with valves propped apart, in distilled water for 15- or 16-hour periods underwent a drop in tissue chloride to values of 0.226 per cent or even 0.112 per cent. Under these conditions animals, although not quite dead, were definitely moribund, and failed to recover when placed in running sea water.

Tolerance by the Mussel of Gradually Altered Concentrations of Sea Salts

While the immediately preceding experiments and earlier investigations seemed to define fairly well the limits of hypo- and hypertonic solutions withstood by mussels following *sudden* immersion, there were grounds for believing that *gradual* changes in the salt concentration in the animals' environment might not bring about signs of injury until greater extremes in both directions were reached.

In order to investigate this question, and to determine if possible the extremes of tissue chloride lost or gained by surviving animals, the following experiments were conducted. Two sets of mussels in separate jars of normal sea water, continuously aerated, were subjected to a gradual change in the chloride concentration of the water, one jar being diluted, the other concentrated daily by slow steps over a period of about six weeks. This gradual change was brought about in the following manner. From one jar containing about eight liters of sea water (seven mussels) some 250 ml. were withdrawn, discarded and replaced by an equal quantity of distilled water daily; from the other jar (eight mussels) the same volume of water was discarded and replaced by an equal quantity of water containing sea salts concentrated by about four-fold (i.e. Cl concentration of 7.725 per cent). These changes brought about a gradual drop in Cl from 1.86 to 0.625 per cent in the first, and a gradual rise from the same initial value to 3.48 per cent in the second solution, over the experimental period. Under these special conditions the mussels survived exposure to previously unexpected concentrations at both extremes.

Table II shows the results of chloride analyses, and a restoration of experimental animals to normal chloride levels after exposure to the gradually altered solutions during five and six-week periods.

It is assumed from the foregoing experiments that it has been possible to ascertain the approximate thresholds of tolerance of the mussel toward the osmotic effects of both hypotonic and hypertonic solutions of

Table II

Limits of tolerated changes in tissue chloride level.

Date 1937	Approximate time elapsed	Cl in hypotonic water	Cl in mussels	Cl in hypertonic water	Cl in mussels
		per cent		*per cent*	
Dec. 13, 14	5 weeks	0.84	3 animals: 0.41% 0.40 0.33 Average: 0.38%	3.09	5 animals: 2.08% 1.82 1.77 1.79 1.72 Average: 1.84%
Dec. 15	5 weeks	0.84	1 animal from same group, placed in running sea water 6 days, and analyzed: Cl = 1.1%	3.09	1 animal from same group, placed in running sea water 6 days, and analyzed: Cl = 0.935%
Dec. 20	6 weeks	0.625	0.26% (sluggish)	—	—
Dec. 21	6 weeks	0.625	0.53% (moribund) Remaining animal in this group placed in running sea water 13 days, and analyzed: Cl = 0.97%	—	—
Dec. 22	6 weeks	—	—	3.48	2 remaining animals (becoming sluggish) 2.19% 2.32 Average: 2.25%

sea salts. It seemed hardly likely, however, that individuals kept in solutions of concentrations below 0.94 per cent Cl or above 2.8 per cent Cl would survive indefinitely, since the flesh of animals in solutions beyond these respective concentrations appeared rather thin, sometimes even emaciated. Furthermore, they failed, in these respective realms of salt concentrations to maintain their constant ratio of tissue chloride to sea water chloride as illustrated in Figure 4. This graph summarizes much of the information reported above and indicates the relationship which exists between the chloride concentration in the surrounding media and that in the tissues of animals immersed therein. Each point represents the average of the analyses of a number of animals immersed in the respective solutions for 24 hours or more, save in the cases of the two lowest points on the hypotonic side, which represent analyses of two respective animals: a single survivor containing 0.26 per cent Cl and the animal which showed the highest Cl value (0.226 per cent) of the group that succumbed to immersion in distilled water (at zero abscissal value). The maintenance of a ratio of 1:1.89 between internal and external chloride is noted in the straight line between the latter concentrations of 0.94 per cent and 2.8 per cent Cl. The projection of this straight line as a dotted line beyond these values shows, when com-

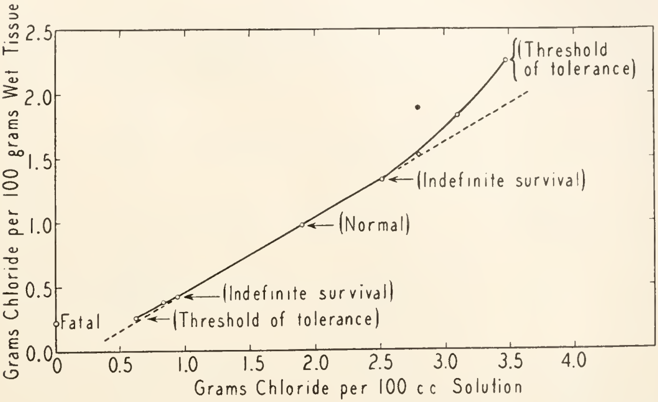

FIG. 4. Relationship between internal and external chloride, in grams Cl per 100 grams tissue vs. grams Cl per 100 ml. water respectively. Points lying between abscissal values of 0.95 and 2.8 represent results obtained after 24 hours or more following *sudden* immersion. Portions of the graph lying below and above these respective values represent the trend taken following gradual alteration of Cl concentration in the water over protracted periods, excepting wherein *distilled* water was used (see text).

pared with the actual curve, the degree of departure in the animals' maintenance of such a ratio. The final two points at each extremity of the curve represent Cl values attained by mussels after *gradual* changes in Cl concentration of the water to the corresponding values shown on the abscissa. (See Table II.)

Content of Water and Chloride in Various Tissues: Exchanges of both Water and Chloride Ions between Tissue Fluid and Environment

Since some question arose as to whether a considerable part of the observed changes in tissue Cl might be assignable to the mere presence of the medium itself in the capillary tubes of the gill structures, analyses were made of water and chloride in (1) the gills and (2) the rest of the tissues en masse, of six mussels taken directly from stock tanks of running sea water. In consistency with the experiment, tissues could not in this case be rinsed in alcohol prior to analysis (hence the slightly elevated Cl values). Table III shows the results of these analyses, and brings out the fact that the gills, as dissected for analysis, possess only a slightly higher moisture content than do the other tissues; furthermore while the chloride content of the gills was about 23 per cent higher than that of the other tissues, their relative proportion of the total wet body weight was only about 15.6 per cent, so that they were responsible

TABLE III

Water and chloride contents of gills and bodies (minus gills) of normal mussels.

Gills:	Wet weight	11.02 grams
	Dry weight	1.09 "
	H_2O	90.1%
	Cl (wet wt.)	1.32%
	Mols. Cl per liter tissue water	0.413
Bodies minus Gills:	Wet weight	59.25
	Dry weight	7.30
	H_2O	87.7%
	Cl (wet weight)	1.075%
	Mols. Cl per liter tissue water	0.345
Combined Total:	Wet weight	70.27
	Dry weight	8.39
	H_2O	88.06%
	Cl	1.11%
	Mols. Cl per liter tissue water	0.355

TABLE IV

Water and chloride contents of foot and body tissues (minus foot) of mussels immersed for 25 hours in sea water of three concentrations. N series: normal sea water, Cl = 1.86 per cent; D series: diluted sea water, Cl = 0.97 per cent; C series: concentrated sea water, Cl = 2.80 per cent.

No.	Sex	Wet wt. foot	Wet wt. other tissues	Combined wet wt.	Dry wt. foot	Dry wt. other tissues	Combined dry wt.	Percentage H_2O foot	Percentage H_2O other tissues	Percentage H_2O whole body	Percentage Cl		
											foot	body	whole
N 1	? immat.	0.27	10.01	10.28	0.06	1.36	1.42	77.8	86.4	86.2	.765	1.06	1.05
N 2	♂ (?)	0.255	12.83	13.09	0.06	1.72	1.78	76.5	86.6	86.4	.71	1.11	1.10
N 3	♀	0.24	10.12	10.36	0.06	1.44	1.50	75.0	85.8	85.5	.57	1.04	1.03
N 4	♂	0.20	9.72	9.92	0.05	1.36	1.41	75.0	86.0	85.8	—	1.10	—
N 5	♂	0.18	7.94	8.12	0.04	1.02	1.06	77.8	87.15	86.95	.89	1.10	1.09
N 6	♀	0.12	10.80	10.92	0.03	1.35	1.38	75.0	87.5	87.4	.66	1.10	1.10
				av.wt. = 10.45				av. = 76.1	av. = 86.6	av. = 86.4	av. = .72	av. = 1.09	av. = 1.08
D 1	♀	0.23	13.07	13.30	0.04	1.275	1.315	82.6	90.25	90.1	.32	0.51	.505
D 2	♀	0.43	15.03	15.46	0.07	1.51	1.58	83.7	89.95	89.8	.38	.48	.48
D 3	♂	0.365	13.10	13.465	0.065	1.64	1.705	82.2	87.5	87.3	.345	.50	.50
D 4	♂	0.50	15.79	16.29	0.08	1.60	1.68	84.0	89.9	89.7	.36	.51	.505
D 5	♂	0.43	14.45	14.88	0.07	1.65	1.72	83.7	88.6	88.4	.39	.465	.46
D 6	? immat.	0.255	10.78	11.035	0.04	1.11	1.15	84.3	89.7	89.6	.32	.50	.50
				av.wt. = 13.07				av. = 83.4	av. = 89.3	av. = 89.15	av. = .35	av. = .49	av. = .49
C 1	? immat.	0.19	10.96	11.15	0.05	1.58	1.63	73.7	85.6	85.4	1.15	1.60	1.59
C 2	♂	0.105	8.96	9.065	0.025	1.34	1.365	76.2	85.05	84.95	1.715	1.60	1.60
C 3	♀	0.365	8.31	8.675	0.10	1.41	1.51	72.6	83.1	82.6	0.96	1.50	1.48
C 4	♂	0.30	9.27	9.57	0.07	1.37	1.44	76.7	85.2	84.95	1.46	1.62	1.61
C 5	♂	0.405	11.77	12.175	0.10	1.63	1.73	75.3	86.15	85.8	1.15	1.42	1.41
C 6	♀	—	—	12.54	—	—	1.90	—	—	84.85		1.34	1.34
	(Foot atrophied to tiny nub)			av.wt. = 10.53				av. = 74.9	av. = 85.05	av. = 84.75	av. = 1.29	av. = 1.51	av. = 1.505

for bringing the total tissue chloride up from 1.075 per cent to 1.11 per cent, an increase of only about 3.5 per cent. Observed shifts in tissue chloride were therefore not due to environmental solution mechanically suspended in the gills.

The question of whether the change in tissue chloride concentration might be due, at least in part, to the osmotic interchange of major quantities of *water,* with or without the migration of Cl ions as well, was investigated in an experiment involving eighteen animals of the usual range of length (i.e. 92 to 110 mm.), the results of which appear in Table IV. Six mussels were immersed in ordinary sea water, six propped open in hypotonic water (" 50 per cent sea water "; 0.97 per cent Cl), and a third set of six propped open in hypertonic water wherein the Cl concentration was 2.80 per cent. After being kept in the respective, constantly aerated solutions for 25 hours, the animals were analyzed. The foot of each was severed with a sharp razor blade and analyzed separately from the other tissues, in order to determine whether such a relatively compact structure might show any considerable difference in chloride shift as compared with that of softer tissues. The dry weights of all tissues were obtained by keeping them overnight in tared containers placed in an electric oven at 105° C., then re-weighing; chloride was determined in the usual manner. In the table, the N series represent the normal control animals kept in sea water, the D series those placed in the diluted sea water, and the C series those immersed in the more concentrated solutions. Some significant trends are observed. The average water content of foot tissues and of whole bodies shows a consistent increase in the order which would have been expected from a consideration of the osmotic effects of the relative solutions, i.e., D > N > C; the relative chloride values of foot tissues or of whole bodies show the opposite order, i.e., C > N > D.

TABLE V

Weight of water and of chloride per unit weight of dry tissue; concentration of chloride per 100 grams of tissue water.

Series	Grams H_2O per gm. dry tissues			Grams Cl per gm. dry flesh			Grams Cl per 100 gm. tissue-water		
	foot	body	whole	foot	body	whole	foot	body	whole
N	3.18	6.46	6.35	0.030	0.081	0.079	0.94	1.25	1.24
D	5.025	8.34	8.22	0.021	0.046	0.045	0.42	0.55	0.55
C	2.98	5.69	5.55	0.051	0.101	0.099	1.72	1.77	1.78

The exchanges of both water and chloride ions, under the conditions of the preceding experiment, are brought out in a quantitative way in Table V, showing for each of the three series, the ratios of average water content and average chloride content, in grams per gram of dry tissue.

Furthermore, by plotting the values for Cl concentration in grams per 100 grams of tissue water, against Cl concentrations in grams per 100 ml. of sea water solutions, we arrive at the nearly linear relationships shown in Fig. 5. The foot, which contains less water, less chloride, and lower concentration of the latter, normally constitutes only about 2 per cent of the total body weight, and exerts no significant influences upon the data collected from analyses of the whole body. Nevertheless, this compact muscular tissue is observed to exchange both water and chloride with the aqueous environment in a manner similar to that of the other tissues.

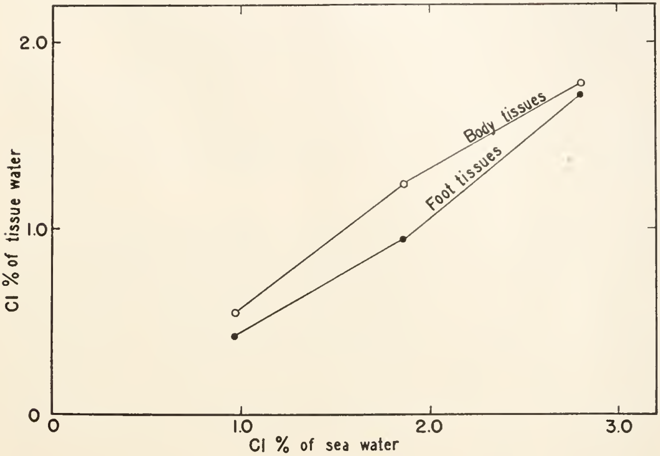

Fig. 5. Relationship between internal chloride concentrations in grams per cent of tissue water and in environmental water respectively. Sea water chlorinities lie within the range of values tolerated by mussels on sudden immersion.

Under the conditions of the above experiments, the mussel alters, throughout a physiologically wide range, the concentrations of its dissolved salts in such manner as to maintain a rather constant ratio between the Cl concentration of internal and that of external water, this ratio appearing to have an average value of the order of 1 : 1.60. The migration of both chloride and water occurs between the tissues and the solution outside.

Discussion

The ready exchange of water and chloride between the mussels' flesh and the environment may not involve any such profound changes in tissue cells themselves as might seem to be the case. The tissues of some organisms contain major quantities of the total chloride in the interstitial fluid, the cells themselves possessing the ion in very small amounts, and not readily undergoing alterations in water content or chloride concentration. This seems to be especially true in certain muscular tissues of mammals (Eggleton, Eggleton and Hamilton, 1937; Bourdillon, 1937), but some investigators are of the opinion that in other tissues (notably in frogs) considerable portion of the chloride is intracellular (Amberson, Nash, Mulder and Binns, 1938).

Since the completion of the present work, some informative results have been published by Steinbach (1940 *a, b*), who studied the content and distribution of water and electrolytes in the excised retractor muscles of certain marine invertebrates, i.e. the holothurian, *Thione briareus* and a sipunculid worm, *Phascolosoma.*

Steinbach immersed his material for a few hours in solutions containing chloride in concentrations of from 52 milli-equivalents per cent (i.e. full-strength sea-water) to nearly zero, employing isosmotic sucrose, or alternatively $NaNO_3$ solution as the sea water diluent. No hypertonic solutions were used. He obtained linear relationships throughout the experimental range of concentrations employed, the fresh *Thyone* tissue being richer in Cl (20.4 meq. Cl per cent) than that of *Phascolosoma* (9.1 meq. Cl per cent when fresh, and 16 meq. Cl per cent when soaked in sea water), and the former yielding steeper slopes of linear change. The normal water-content was very similar in both species, i.e. 75.9 per cent in *Thyone* and 78 per cent in *Phascolosoma* muscle.

The present writer immersed whole mussels for 24 hours or longer in normal, dilute, and concentrated solutions of natural sea salts within the range successfully tolerated by the animals. Linear relationships were apparent between the range of about 1.74 and 79.1 meq. Cl per cent of environmental water, when dealing with whole bodies, while such data as were collected upon the subsequently amputated foot alone revealed a nearly linear function between environmental Cl concentrations of 27.7 and 79.1 meq. Cl per cent.

It is also of interest to note that the initial concentration of Cl in Steinbach's *Thyone* muscle is virtually identical with that of the muscular foot of *Mytilus,* viz.; 20.4 and 20.3 meq. Cl per cent respectively, and that the water-contents of both are close, viz.: 75.9 per cent and 76.1 per cent. Furthermore, the Cl concentration in half-strength sea water

(Steinbach's fortified with isosmotic solutions of sucrose or $NaNO_3$, mine merely diluted) resulted in a decrease in *Thyone* muscle Cl to a value close to that determined for *Mytilus*-foot, viz.; 12 vs. 9.9 meq. Cl per cent respectively.

Steinbach's experiments led him to conclude that muscular tissues of both *Thyone* and *Phascolosoma* (classed as smooth muscle) contain sodium chloride almost exclusively in the extracellular space, that it is free to equilibrate by simple diffusion with external solutions, and that little if any can penetrate the tissue-cells themselves. He discusses the evidence in support of the conclusion that virtually all chloride is extracellular in the material studied by him, and in striated muscles of frogs and some other vertebrates as well, and that measurement of chloride content of such tissues may be employed as a relative measure of the extracellular space.

The muscular foot of *Mytilus* is observed to show osmotic behavior closely similar to that of the retractor muscles of *Thyone* and *Phascolosoma*, and must be very like the latter tissues in biochemical constitution and function.

The whole body of *Mytilus* shows a similar linear slope as does the foot tissue within the range of environmental Cl concentrations compared, but exhibits consistently higher water and Cl content. Deviations from the linear relationship between whole tissue Cl and sea water Cl which occur at extreme dilutions and extreme concentrations represent the failure of the physiological mechanism to control any longer the integrity of the chloride-free cellular space under relatively drastic conditions.

Investigations of biochemical or physiological changes in tissues of whole organisms, living successfully in controlled foreign environments, allow, in the conclusions drawn, a degree of certainty less frequently assured from data collected on isolated, surviving tissues. In the present instance, it is of much interest to note some close parallelisms between different species investigated by the respective experimental approaches.

The work reported in this paper was begun with the experimental ecological viewpoint in mind. In summarizing briefly, it is recalled that while the adult California mussel is able to adjust itself, with accompanying changes in tissue constitution, to a considerable range of salinities in the laboratory, this species, unlike *M. edulis,* is rarely if ever found in bays and estuaries, even though such waters may be considerably less diluted than were solutions which the animals have been shown experimentally to tolerate for indefinite periods.

Our findings during the course of this work suggested some experiments on the effect of dilute solutions of sea salts upon ripe sperm and eggs of this species, the process of fertilization, and subsequent development. Some preliminary experiments of this kind were accordingly carried out at the Scripps Institution by Dr. Robert T. Young, whose findings indicate that sea water diluted by more than 25 per cent may exert injurious effects upon (1) the sex products themselves, (2) incidence of fertilization, and (3) subsequent development. Doubtless the dilution of water in bays and estuaries is not the only factor responsible for the failure of *Mytilus californianus* to colonize them, but the sensitivity of eggs, sperm, and larvae to the dilute environments provides one tangible clue which should prove helpful in further attack on this and kindred problems.

Acknowledgments

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Summary

1. Experiments indicate that the adult mussel *Mytilus californiaus* is heterosmotic, yet potentially euryhaline to a considerable degree, although sperm, eggs and larvae are highly stenohaline toward dilution of the environment.

2. The tissue-chloride content is close to 1 per cent by wet weight, varying only slightly with season. Mature males show slightly higher chloride values than do the mature females, due to the higher chloride content of testicular tissues than of ovarian tissues.

3. Mussels can survive for indefinite periods the sudden and continued exposure of their tissues to sea water diluted by 50 per cent (Cl conc. 0.94 per cent) or water concentrated to half again its normal value (Cl conc. 2.73 per cent to 2.8 per cent). Below or above these two respective extremes, sudden immersion is fatal.

4. Within the limits of the physiologically tolerated range indicated, the concentrations of tissue chloride are adjusted to concentrations of chloride in the environment, with maintenance of an approximate value of 1 : 1.60, calculated as grams per 100 ml. of internal and external water.

5. Considerable individual differences exist in the rate of establishment of equilibrium between environmental and tissue chloride concentrations, when mussels are exposed to the indicated dilute and concentrated solutions.

6. While sudden immersion in solutions of sea salts below or above the respective limits resulted fatally, it was possible for mussels to survive in solutions considerably beyond such limits, i.e. in water diluted to as low as 0.62 per cent Cl, or concentrated to 3.48 per cent Cl, if the concentrations were altered by *gradual steps.*

7. Mussels surviving in sea water, gradually diluted to a chlorinity of 0.625 per cent Cl, underwent a fall in their tissue chloride to values of about 0.26 per cent to 0.38 per cent; animals kept in sea water gradually concentrated to a chlorinity of 3.48 per cent underwent a rise in their tissue chloride to average values of 2.25 per cent; at these respective points animals were at their threshold of tolerance and showed incipient sluggishness. Animals of such extreme chloride levels, however, recovered if placed in running sea water, and readily underwent therein a restoration of their tissue chlorides to normal values.

8. Exposure of mussels to the diluted or concentrated solutions results in migrations of both water and chloride between internal and external media.

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MATING REACTIONS OF ENUCLEATE FRAGMENTS IN PARAMECIUM BURSARIA

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INTRODUCTION

The presence of distinct mating types in *Paramecium* has been demonstrated by several investigators (Sonneborn, 1937; Kimball, 1937; Jennings, 1938; Sonneborn, 1938; and Jennings, 1939). Under appropriate conditions individuals belonging to different mating types in the same "group" will, when they are placed together, immediately agglutinate and later form pairs. Such agglutination has been called the "mating reaction." The present study is designed to answer the question: Do enucleate fragments of *Paramecium* manifest mating reactions?

In this investigation we have studied, for comparison, the following phenomena: (1) mating reaction between whole animals, (2) mating reaction between nucleate [1] fragments and whole animals, (3) mating reaction between enucleate [1] fragments and whole animals, (4) mating reaction between enucleate fragments. The results of these comparative studies will be reported in the order given.

Preliminary work on this problem was originally begun at the Osborn Zoölogical Laboratory, Yale University, in collaboration with Dr. R. F. Kimball; but circumstances unfortunately prevented its completion there. The present study was carried on largely at the University of California at Los Angeles and completed at the University of Vermont. A preliminary report appeared in *Science,* 91: 246 (1940).

MATERIAL AND METHODS

Paramecium bursaria—the green Paramecium—is especially favorable for this study for several reasons. (1) As far as we know, *bursaria* is the only species of *Paramecium* in which enucleate fragments are viable. (2) The tendency of animals of this species to creep slowly over the bottom of the container facilitates cutting with a glass needle to such an extent that large numbers of fragments can be obtained.

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[1] In this paper the word "nucleate" is used to indicate the presence of both the macronucleus and the micronucleus; the word "enucleate" meaning the absence of both nuclei.

(3) The single micronucleus of this species (especially in the races used in the present investigation) is large and stains deeply with hematoxylin. (4) Stability of mating type permits one to obtain uniform and constant material for study.

For this study two races of *P. bursaria*—McD_3 and *Gr*14—were used.[2] They belong to two different mating types of Group II (Jennings, 1939). Under suitable conditions they give a marked mating reaction when placed together, and permanent pairs are later formed.

Race McD_3 was collected near Baltimore, Maryland; race *Gr*14 from south of Greensboro, North Carolina. McD_3 is a large race, while *Gr*14 is somewhat smaller. In both races the micronucleus is large and stains deeply with hematoxylin. After such staining the micronucleus or a piece of macronucleus could thus easily be detected if present in any fragment.

The animals were cultured in essentially the same manner as described by Jennings (1939). A number of cultures of each race were kept in the laboratory, and only those which gave the strongest mating reaction were used.

The animals to be operated upon were placed in a depression slide and cut with a fine glass needle under a dissecting microscope. When the needle passed directly through the mid-region of the animal, the nuclei were usually seen to be extruded from one of the fragments; while if the needle cut to one side of the mid-region one large nucleate and one smaller enucleate fragment resulted. Only fragments one-half the size of the original animal or smaller were isolated for testing since these were most likely to be enucleate.

When a definite mating reaction had been observed, the fragments were fixed in hot Schaudinn's fluid (95 cc. Schaudinn's fluid and 5 cc. glacial acetic acid) or in Bouin's fluid at room temperature. They were stained in iron-hematoxylin, destained in aqueous picric acid, and mounted in damar.

OBSERVATIONS

Mating Reaction between Whole Animals

Jennings (1939) has described in detail the mating reaction between whole animals in *P. bursaria.* We have, however, noted an additional feature. When the area of contact of one animal with another is small there appears a distinct flattening if not an appreciable indentation of that part of the cell (Fig. 1). At present an explanation of this phenomenon cannot be given, but at least the response indicates that the union between the animals involves more than a possible adhesion of the cilia. It is significant that if members of a reacting pair (two whole animals, or a whole animal and a fragment) are gently separated with the glass needle, these flattenings or indentations (Fig. 3, *g–i*) do not disappear at once but only gradually round out to the normal contour.

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[2] We are indebted to Professor H. S. Jennings for these two races of *P. bursaria.*

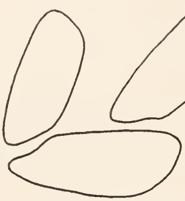

FIG. 1. Mating reaction of whole animals. Two individuals in this case have become attached to a third animal. In each animal there appears a flattening of the contour of the body at the region of contact. (This drawing and others to follow are free-hand sketches from the living material.)

Mating Reactions between Nucleate Fragments and Whole Animals

When *P. bursaria* is cut transversely with the needle there usually results one larger and one smaller fragment. It is the larger fragment which contains the nuclei, the nuclear complex being visible as a clear sphere in the living fragments. Shortly after cutting, such nucleate fragments of McD_3 were placed with whole animals of *Gr*14. The mating reaction took place at once, and on the following day intimate fusion of fragments with whole animals, similar to conjugation between whole animals, was observed. Conversely, nucleate fragments of *Gr*14 were placed with whole animals of McD_3, and such mixtures gave the same mating reaction and intimate fusion (Fig. 2) as described above. (Samples of such pairs were stained with aceto-carmine and a full nuclear complement was invariably found in the fragment.) It has not

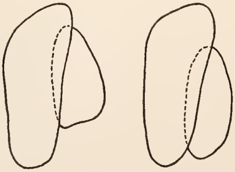

FIG. 2. Large nucleate fragments of *Gr*14 fused with McD_3 whole animals.

yet been determined whether the intimate fusion between the nucleate fragment and the whole animal initiates nuclear changes in either or both partners.

It should also be noted that the nucleate fragments not only exhibited mating reactions immediately but also on the second and third days after cutting. After the mixture was made and mating reaction noted, it was placed aside undisturbed in a moist chamber. This mixture was again examined on the second and third days following. Such nucleate fragments as had not already fused in conjugation were again found to be exhibiting the mating reaction. Apparently the nucleate fragments which did not fuse with the whole animals gave repeated mating reactions.[3] Thus the nucleate fragments can react not only on the day when they are prepared, but also on subsequent days; and this is quite in contrast to enucleate fragments which give the mating reaction on the day of cutting but never subsequently, though they may be active two or more days later (see below).

Viability of Enucleate Fragments

Enucleate fragments of *P. bursaria* may remain alive for as long as four days, and during that time exhibit a surprisingly normal behavior, swimming actively in one direction, alternating the direction of movement, spiralling, and coming to rest adjacent to masses of food (Tartar, 1938). Observations on 100 enucleate fragments each of McD_3 and $Gr14$ showed that practically 100 per cent of such fragments were alive and active after one day, and 50 per cent after two days. Such hardiness of enucleate fragments made possible the investigation herein described. In the present study the enucleate fragments were tested within half an hour after cutting.

Mating Reaction between Enucleate Fragments and Whole Animals

Enucleate fragments of either race were found to give the mating reaction with whole animals of the other race (Fig. 3, *a–f*). Enucleate fragments never agglutinate with fragments or whole animals of the same race. The mating reaction was observed in 131 small enucleate fragments of McD_3 mated with whole animals of $Gr14$. A sample of fifteen of these fragments which reacted was fixed in Schaudinn's fluid and stained with iron-hematoxylin to test whether nuclei or parts thereof might be present in the fragments. In no case was a micronucleus or a piece of macronucleus found in a fragment.

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[3] The races studied show a diurnal reactivity, discontinuing mating reactions in the late afternoon and not reacting again until the morning of the following day.

In the reciprocal cross enucleate fragments of *Gr*14 were placed with whole animals of McD_3. Such a mixture gave the same agglutination as described above. Twelve such fragments which reacted were stained and no trace of a nucleus or part of a nucleus was found in them.

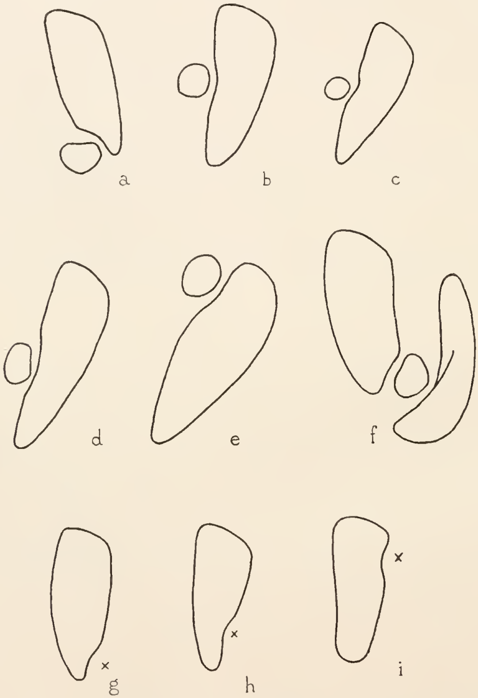

FIG. 3. Mating reactions between enucleate fragments and whole animals. Note the flattening or indentation of the body of the animal at the point of union with a fragment (Fig. 3, *a–f*). Whole animals which were separated from their attachment with enucleate fragments still retain typical indentation (X) at the points of union (Fig. 3, *g–i*).

Apparently in no respect does the mating reaction between enucleate fragments and whole animals differ from that between whole animals. Such similarity is shown by the following observations: (1) The reacting fragment agglutinates with the first animal of the other race with which it happens to collide. Subsequently, one, two, three or more animals of the other race may become attached to the fragment, thus forming the typical agglutinated clump (Fig. 3*f*). (2) The fragment remains securely attached to the whole animal, i.e., the two do not rotate upon one another. The direct medium of this union is not the surfaces of the fragment and the whole animal since the partners remain separated by the distance of their cilia. Yet the union is more intimate than might be suspected, for at whatever region the fragment attaches to the whole animal, a flattening or slight indentation of the normal contour of the whole animal is there produced (Fig. 3). (3) The pairs or clumps formed by the agglutination of fragments and whole animals break up at approximately the same time as that at which clumps of whole animals mated simultaneously break up into conjugating pairs and single animals. This latter point is the conclusion from a separate study of the duration of agglutination of enucleate fragments with whole animals in which two additional groups of McD_3 enucleate fragments (total number, 90) were mated with *Gr*14 whole animals. All enucleate fragments became separated from the whole animals 123 to 140 minutes after the beginning of agglutination and never again united with them. This was approximately the time required for simultaneously mixed whole animals, which formed large clumps, to break up into pairs and single animals (123 to 153 minutes).

The mating reaction between enucleate fragments and whole animals is thus in these respects altogether normal, *but it was never followed by true fusion as in conjugation.* It remains to be determined whether contact with the enucleate fragment is sufficient stimulus to initiate nuclear changes in the whole animals.

Although mating reaction is thus shown by enucleate fragments tested promptly after cutting, this is not the case if the enucleate fragments are kept for 24 hours before they are mixed with whole animals of the other race. Enucleate fragments from a McD_3 culture were prepared at the time when tests showed the animals of this culture to be most reactive with *Gr*14. These McD_3 enucleate fragments were placed with *Gr*14 whole animals 24 hours after they were cut. None of the 115 fragments so tested in delayed mixing showed any mating reaction although they were alive and active. At the time of mixing the enucleate fragments with the whole animals, control experiments showed that the McD_3 whole animals from the culture in question were again strongly reactive.

Mating Reaction between Enucleate Fragments

For a study of reaction between enucleate fragments, one fragment of each race was placed in a very small drop of culture fluid and the pair observed. In approximately half of the cases the enucleate fragments agglutinated at once when, during their rapid movements, they first collided with one another (Fig. 4). Mating reaction between enucleate fragments was under these conditions apparently not so strong as between enucleate fragments and whole animals, for the pairs were easily separated by jarring, and even when not disturbed they remained attached for not longer than ten minutes. That the response is not a mere chance adherence, however, is shown by the fact that it never occurred between fragments of the same race even though these were observed to collide with one another. Although flattening at the point of contact was not observed in these small fragments, they swam together as one animal without rotating upon one another, a behavior typical of the mating reaction. After the two reacting fragments separated, they frequently again agglutinated and became attached.

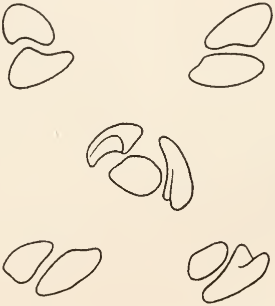

FIG. 4. Mating reaction between enucleate fragments. (These fragments were later fixed and stained and were found to be enucleate.)

Thirty enucleate fragments (15 of each race) were tested as described; and of these 16 gave the mating reaction. Each pair of reacting fragments was separately fixed and stained in iron-hematoxylin. In no case was a nucleus or part of a nucleus found in any of the fragments.

Another method of observing the mating reactions between enucleate fragments consists of introducing under the dissecting microscope one fragment of one race into a drop containing several fragments of the other race and to watch the introduced fragment continuously. This procedure greatly increases the probability of the fragment finding a partner. Under these circumstances two more cases of mating reaction were observed: in one case the introduced McD_3 fragment remained attached to two *Gr*14 fragments for five minutes; in the other case a McD_3 fragment paired with a *Gr*14 fragment for twelve minutes.

Still another method was to place three to six enucleate fragments of one race (*Gr*14) into a drop containing many enucleate fragments of the other race (McD_3). Out of a total of 22 *Gr*14 fragments so tested, 15 reacted. Typical mating reaction occurred; pairs of enucleate fragments and groups of three enucleate fragments were observed. When, as here, the drop of water in which the reaction is followed is of sufficient size that evaporation does not interfere and the reacting fragments need not be disturbed by replenishment of the drop, the conditions may be said to be most nearly normal; and under these circumstances pairs of enucleate fragments and groups of three enucleate fragments were found to remain continuously united in the mating reaction for as long as 34 minutes.

Thus a total of 25 cases of mating reaction between enucleate fragments has been recorded. Of these, 12 sample pairs of fragments were carefully stained and no nucleus found in either fragment. In every experiment, it is to be emphasized, the unmixed enucleate fragments of each race were conscientiously watched en masse, and in no instance did fragments of the same race react with one another though collisions between them were frequent.

Thus the cytoplasm alone (in the absence of the nuclei) exhibits the reactivity and diversity of mating type. Of course, this reactivity may be due to the retention of influence of the nuclei which have just been removed. This possibility is strongly suggested by the fact that enucleate fragments lose their reactivity within a day and do not regain it thereafter.

SUMMARY

1. Comparative studies were made on the following phenomena in two races of *Paramecium bursaria* belonging to two different mating types: (*a*) the mating reaction between whole animals, (*b*) the mating reaction between nucleate fragments and whole animals, (*c*) the mating reaction between enucleate fragments and whole animals, and (*d*) the mating reaction between enucleate fragments.

2. In the mating reaction between whole animals a phenomenon hitherto unreported was observed: When two animals become attached there is a flattening or even an appreciable indentation at the point of union which is most striking when the area of contact is small.

3. Nucleate fragments of either race show mating reaction with whole animals belonging to the other race. The mating reaction may be followed by an intimate fusion, as in conjugation between whole animals, between the nucleate fragment and whole animal.

4. Enucleate fragments of either race give the mating reaction with whole animals of the other race. Mating reactions never occur between enucleate fragments and whole animals of the same race. Mating reaction between enucleate fragments and whole animals appears to be identical with that between whole animals, but it was never followed by intimate fusion as in conjugation between whole animals.

5. The mating reaction also occurs between enucleate fragments belonging to two different races. Controls showed that mating reactions never occur between enucleate fragments of the same race.

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THE TRANSPORT OF CO_2 IN THE BLOOD OF CERTAIN FRESHWATER FISHES

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The effect of carbon dioxide (and other acids) on the affinity of the hemoglobin in fish blood for oxygen varies greatly from species to species (Redfield, 1933). In some species not only the affinity of the blood for oxygen, but the oxygen capacity too, is greatly diminished by relatively low pressures of CO_2 (Root, 1931). The physiological and ecological implications of great differences in affinity for oxygen and sensitivity to CO_2 have become more apparent as a result of a series of studies on freshwater fish, which are summarized in a recent paper by Black (1940). A few of the findings may be recapitulated briefly as follows. Carbon dioxide decreases the affinity of the blood for oxygen to a greater degree in those fish inhabiting deeper and colder water. The same bloods have also a lower affinity for oxygen at minimal pressures of CO_2. These two characteristics would act to offset the effect of low temperatures, which is to lower the pressure at which oxygen is available to the tissues. Another manner in which sensitivity to CO_2 may be useful to fish inhabiting deep water, is in the regulation of buoyancy at different depths, by facilitating the formation in the swimbladder of gases rich in oxygen, as suggested by Haldane (1922) and Hall (1924).

The present study was designed with two objects in mind. The first was to investigate the mechanisms by which the great differences in sensitivity to CO_2 are achieved. The second was to determine the probable range of physiological tensions of CO_2 in a series of freshwater fish comprising some of those used in the foregoing studies. This information is difficult to obtain with any degree of accuracy in fish, but even approximate determinations may be of use in evaluating some of the relationships described above.

In pursuit of the first object, a detailed study of the characteristics of CO_2 transport was made on the blood of two species differing widely in the sensitivity of their blood to CO_2, namely rainbow trout, *Salmo gairdnerii* Richardson, and carp, *Cyprinus carpio* Linnaeus. Trout are among the fish most sensitive to CO_2 and carp, while not the most insensitive, are relatively so. Availability in sufficient numbers to supply enough blood was an important consideration in the choice of these species. Data on physiological CO_2 tensions were obtained on the same species as well as on a few specimens of bullhead, *Ameiurus nebulosus* Le Sueur, and sucker, *Catostomus commersonnii* Lacepède, which happened to be available. The blood of the bullhead is even less sensitive to CO_2 than that of the carp, while those of the sucker and the trout are of about the same order of sensitivity.

METHODS

Blood was drawn from the heart into a syringe containing heparin. No fluoride or oxalate was used, since these have been found to cause progressive swelling of the erythrocytes and eventual hemolysis in certain fish bloods (Black and Irving, 1938; Hamdi and Ferguson, 1940). For the construction of dissociation curves it was necessary to pool the blood from several fish. Equilibration was done at 15° C., except in specified cases. The blood was kept on ice throughout the day. Samples were equilibrated one at a time for fifteen minutes in tonometers of the original Barcroft type (1914). A centrifuge tube was attached to the open end of the tonometer by rubber tubing. After equilibration the blood was collected in the centrifuge tube and separated from the gas phase by clamping the rubber tubing. The gas was analyzed for CO_2 and O_2 in a Haldane apparatus. The blood in the centrifuge tube was covered with liquid paraffin and samples were removed without delay for analysis of CO_2 and O_2 by the manometric method of Van Slyke; of chloride by the open Carius method; and of water content by drying at 105° C. Another sample was centrifuged in a capillary tube for twenty minutes at a centrifugal force of about 3000 times gravity for the estimation of packed cell volume. The rest of the blood was also centrifuged under oil for 20 minutes and samples of plasma removed for the analyses listed above. A number of experiments were performed to evaluate the error introduced by metabolism of the blood during the foregoing procedures. Hourly analyses were done on blood kept in sealed syringes at 15° C. and on ice. Trout blood was found to have a somewhat higher metabolic rate than carp blood, and the highest rate found in trout blood at 15° C. was 1 cc. of oxygen consumed and 1 cc. of CO_2 produced per 100 cc. blood per hour. The only estimation which might have been appreciably affected by even this highest metabolic rate was the estimation of cell volume in which centrifuging was done at 25° C. or thereabouts for 20 minutes. It was possible in the later experiments on trout blood to minimize this source of error by reducing the time of centrifuging to 5 minutes in a high speed hematocrit centrifuge. The results with both long and short times of centrifuging were in substantial agreement.

In trout blood kept on ice, the highest metabolic rate was found to be 0.5 cc. per 100 cc. per hour. This figure may be used to estimate the maximum correction for the figures on O_2 and CO_2 content of the venous blood of trout, because the trout blood was kept on ice for three or four hours in transit from the hatchery to the laboratory.

Some experiments were done to determine the change in CO_2 capacity with time. No changes were found in blood kept on ice for as long as 12 hours. When the blood was kept at 10° C. in a refrigerator for 24 to 48 hours, small changes occurred. It is interesting that the CO_2 capacity increased in blood (both carp and trout) kept fully oxygenated, but decreased in blood which was partly or fully reduced, suggesting something like a Pasteur reaction.

The gas content of mixed venous blood, drawn from the heart into an oiled syringe containing heparin, was determined by analysis without exposure to air. Three portions of the same sample were then equilibrated with different gas mixtures and subsequently analyzed. One portion was equilibrated with about 2 mm. CO_2 in air. This gave the oxygen capacity and one point on the CO_2 dissociation curve. Another portion was equilibrated with 8–12 mm. CO_2 in air and the third portion with 8–12 mm. CO_2 in N_2. These gave the positions of the CO_2 dissociation curves of oxygenated and reduced blood respectively. The equilibrations in these particular experiments were done at the temperature of the water from which the fish were taken. Knowing the oxygen content of the sample of venous blood and the oxygen capacity of the same sample, it was possible to estimate the position of the CO_2 dissociation curve of the venous blood between those of the fully oxygenated and fully reduced bloods. Then knowing the CO_2 content of the venous blood, its CO_2 tension could be read off on the abscissa with an error probably not more than 2 or 3 mm. Hg.

During the withdrawal of the venous blood, the gills were not aerated, but only samples which flowed freely into the syringe at the first stab were used. Since even the trout survived this procedure without apparent harm, the estimated tensions may be regarded as well within the limit of tolerance of the fish, though probably above the average for the resting state.

OBSERVATIONS

Physiological Gas Tensions

The results on the gas contents and tensions in circulating blood are presented first, since they indicate the part of the CO_2 dissociation curves of greatest physiological importance. Table I gives the CO_2 content, percentage saturation with oxygen and estimated CO_2 tensions of samples of venous blood drawn from four species of fish. Only the trout blood could have changed significantly between the time of drawing the blood and the time of analysis. If maximum corrections for metabolism are applied to the results on trout blood, the CO_2 tension would be about 1 mm. lower, the CO_2 contents about 2 vols. p.c. lower and the oxygen contents about 2 vols. p.c. higher.

TABLE I

Venous blood gases

Trout	Temperature	CO_2	O_2	P CO_2
	°C.	*v.p.c.*	*per cent sat.*	*mm. Hg*
B	22	21.0	0	9
C	22	24.8	3	10
D	15	19.4	0	8
E	15	22.8	0	10
Carp				
J	10	36.4	47	5
G	15	31.2	30	10
H	15	28.6	18	7
Sucker				
A	8	47.8	37	9
B	8	36.3	26	7
Catfish				
A	8	21.4	62	8

No measurements were made on the aerated blood from the gills, but a rough estimate of the CO_2 tension in aerated blood can be made in the following manner. Trout *D* in Table I is favorable for purposes of illustration, because it had practically the same O_2 and CO_2 capacity as the composite blood *F* in Fig. 1. If the blood of trout *D* were fully oxygenated, it would gain about 11 vols. p.c. of 0. Assuming an R.Q. of unity, the CO_2 lost would be 11 vols. p.c., leaving a CO_2 content of about 8 vols. p.c. A CO_2 content of 8 vols. p.c. corresponds on the curve for oxygenated trout blood in Fig. 1 to a CO_2 tension of about 3.5 mm. Similar calculations for all the bloods in Table I gave estimated

CO_2 tensions of 3–5 mm. for fully oxygenated "arterial" blood. These figures indicate that the most important range of physiological CO_2 pressure is between 3 and 10 mm. Hg in all four species. It is a matter of considerable interest that the physiological range is so similar in fish representing the extremes of variation in the effect of CO_2 on the combination of oxygen in the blood. It may be concluded that the consequences of differences in sensitivity to CO_2 are not evaded to any extent, as might conceivably be the case if different species maintained themselves at significantly different CO_2 pressures.

Another implication of the foregoing results is that CO_2 tensions above 10 mm. could only be attained in any of these fish under conditions of oxygen debt. Higher tensions might occur, however, in certain tissues as a result of acid production locally. Consequently the rather low CO_2 tensions found in mixed venous blood need not be regarded as the maximal CO_2 tensions which may operate in the production of gases in the swim-bladder in deep water.

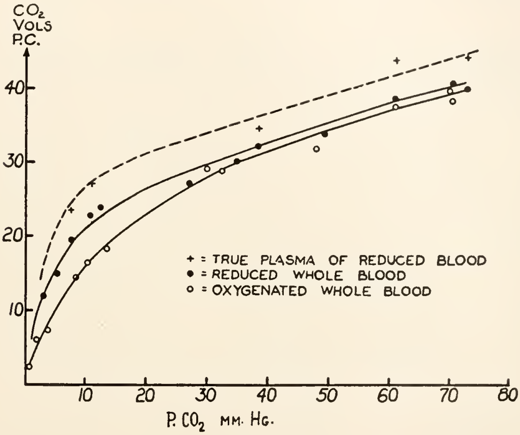

Fig. 1. CO_2 dissociation curves of pooled blood of rainbow trout at 15° C.

CO_2 Dissociation Curves

Figure 1 shows composite curves of two batches of blood each from twelve rainbow trout. They resemble the curves obtained by Root (1931) and Root and Irving (1940) on various marine fish in the convergence of the curves for oxygenated and reduced blood at the higher pressures of CO_2. It can be seen in Table II that the oxygenated blood is not fully oxygenated at the higher pressures of CO_2 even though in some cases it was exposed to pressures of oxygen as high as 670 mm. Hg.

The apparent disappearance of the effect of oxygenation on CO_2 capacity (Haldane effect) at the higher pressures of CO_2 can be due only in part to incomplete oxygenation of the oxygenated blood because even at the high pressures it is still half saturated with O_2.

In Fig. 2 are shown the CO_2 dissociation curves of carp blood. Two sets of curves are given representing the range of variation found in six batches of carp blood. The greater variation shown by the carp may be due in part to the use of fewer fish for each batch of blood (the carp being larger fish), and consequently less averaging out of individual variations. The curves for carp differ from those of the trout in two important respects. Firstly they are higher, indicating a higher pH at a given pressure of CO_2. Secondly the curves of oxygenated and reduced bloods are widely separated and show only a slight tendency to converge at higher CO_2 pressures.

TABLE II

Complete data on blood of trout F at 15°C. To calculate combined O_2 *(Hb* O_2*) the Bunsen solubility coefficient of oxygen in the blood at 15° is assumed to be 0.036. The meaning of r* CO_2 *and r Cl is explained in the text.*

P CO_2	PO_2	CO_2 content		Hb O_2	r CO_2	Cell vol.	Chloride		r Cl	Water content	
		Whole blood	Plasma				Whole blood	Plasma		Whole blood	Plasma
mm. Hg	*mm. Hg*	*v.p.v.*	*v.p.c.*	*v.p.c.*		*per cent*	*m. eq./l.*	*m. eq./l.*		*g./100 g.*	*g./100 g.*
2.2	Air	6.1	8.0	9.9	0.33	32.8	—	140.0	0.60	86.0	94.5
2.7	0	11.9	13.8	0.0	0.71	33.0	116	139.5	0.61	86.0	94.6
10.7	124	16.3	18.0	7.3	0.90	35.3	118	140.0	0.65	86.5	95.5
12.5	0	23.8	26.4	0.0	0.95	47.0	117	136.0	0.81	85.9	94.3
32.7	168	28.6	31.5	5.0	0.94	43.0	116	138.0	0.78	86.0	94.9
38.5	0	31.8	34.2	0.0	1.02	44.0	117	139.5	0.77	85.8	94.5
61.0	675	37.3	40.6	5.5	0.94	37.4	116	139.0	0.68	85.3	94.6
70.5	0	40.4	44.0	0.0	0.98	43.8	116	138.0	0.77	84.8	95.0

The higher CO_2 capacity of carp blood indicates a higher pH which must be attributed to the regulation of the acid-base balance of the fish as a whole at a more alkaline level. The CO_2 dissociation curves of true plasma in both species lie above the curves for whole blood, in this respect resembling mammalian blood rather than dogfish blood (Ferguson, Horvath and Pappenheimer, 1938). They indicate a higher concentration of CO_2 in the plasma than in cells at the same pressure of

CO_2. The distribution of CO_2 between cells and plasma is more precisely indicated in Table II in the column headed r CO_2.

Distribution of Electrolytes

The distribution ratio r CO_2 is the concentration of combined CO_2 per gram of cell water divided by the combined CO_2 per gram of plasma water. Combined CO_2 is calculated by subtraction of the physically dissolved CO_2 from the total CO_2, using the factors 0.125 and 0.105 for plasma and cells respectively, which multiplied by the PCO_2 in mm. Hg give the concentrations of dissolved CO_2 in volumes per cent. RCl is the analogous distribution ratio for chlorides. Little weight should be given to individual values of the distribution ratios because of the large number of possible errors that are introduced in their computation. On the average, however, it is evident that in carp blood the values of r CO_2 do not tend to exceed those of r Cl as they do in mammalian blood where the higher value of r CO_2 is probably due mostly to the presence of carbamino compounds of CO_2 with hemoglobin (Roughton, 1935). In the trout blood the values of r CO_2 do tend to exceed somewhat the values of r Cl. Thus the distribution ratios provide no evidence for the presence of carbamino compounds in the carp erythrocytes, but would be consistent with the presence of a small amount in trout cells. They

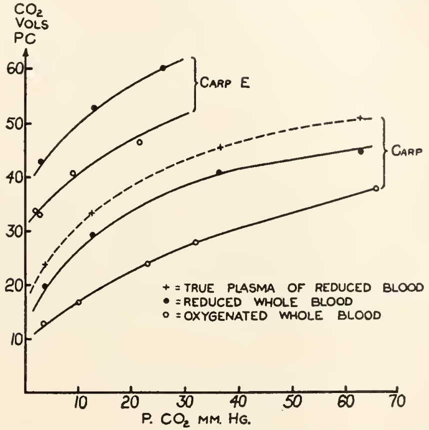

FIG. 2. CO_2 dissociation curves of carp blood at 15° C. Of six batches of pooled carp blood, E had the highest CO_2 capacity and I the lowest.

are consistent with the hypothesis that the combined CO_2 in red cells is largely in the form of bicarbonate and that the bicarbonate and the chloride are partitioned between the red cells and plasma according to a Donnan distribution. The figures on plasma chloride of carp blood in Table III give further evidence of a Donnan equilibrium with chloride and bicarbonate ions diffusible. The plasma chloride decreases regularly (except for one figure) with each increase in plasma CO_2 consistent with a migration of chloride into the cells with increasing acidity. Plasma chloride in trout blood also shows a decrease with increasing CO_2, but in a very irregular fashion which, however, acquires more meaning when considered with the changes in packed cell volume.

TABLE III

Complete data on blood of carp I at 15°C.

P CO_2	PO_2	CO_2 content		Hb O_2	r CO_2	Cell vol.	Chloride		r Cl	Water content	
		Whole blood	Plasma				Whole blood	Plasma		Whole blood	Plasma
mm. Hg	*mm. Hg*	*v.p.c.*	*v.p.c.*	*v.p.c.*		*per cent*	*m. eq./l.*	*m. eq./l.*		*g./100 g.*	*g./100 g.*
3.4	158	12.9	15.2	12.3	0.77	39.6	124.5	146.0	0.94	82.5	94.8
3.7	2.3	19.8	23.8	1.8	0.77	41.2	125.0	144.0	0.88	83.9	94.8
10.5	148	17.0	21.2	12.7	0.61	38.8	125.0	146.5	0.90	84.1	95.1
12.6	0	29.3	33.7	0.4	0.88	40.6	125.5	142.5	0.91	83.5	94.6
32.2	145	27.9	31.6	10.8	0.90	39.5	125.5	144.5	0.85	83.2	95.0
36.6	0	41.1	45.9	0	0.95	40.2	126.0	141.5	0.94	83.4	94.5
66.2	224	38.1	42.2	10.8	0.98	40.9	125.0	142.0	0.92	83.6	94.4
63.2	0	45.0	51.3	0	0.88	41.2	124.5	140.5	0.95	82.7	95.2

Cell Volumes

In Fig. 3 the packed cell volumes of carp and trout blood are plotted against pressure of CO_2. In carp blood the cell volume decreases slightly with the first increment of CO_2 pressure, but with further increase in P CO_2 the volume increases as in mammalian blood, and by about the same amount for each increment of CO_2 combined, namely by about 5 p.c. of their volume for an increase of 10 m.eq. per liter of combined CO_2. The cells of T.J.F. (Henderson, 1928) increased by 6 per cent for an increase of 10 m.eq. of CO_2 combined.

The volume changes for trout blood shown in Fig. 3 are the composite data from three batches of blood. The trout cells show greater changes in volume than do the carp cells. They reach their maximum size between 10–20 mm. Hg of CO_2 pressure. The reduced cells show a much greater increase in volume, but at pressures above 20 mm. Hg they decrease again with increasing P CO_2. It can be seen in Table II that the largest cell volume corresponds with the lowest concentration of chloride in the plasma, suggesting that a migration of chloride from the plasma, presumably into the cells, has occurred. The extent of this migration is depicted in Fig. 4, where the chloride content in the plasma of a liter of blood is plotted against the bicarbonate content of the plasma of a liter of blood. The contents are calculated by multiplying the concentrations per liter of plasma by the fraction of the whole blood volume which is plasma. In carp blood it appears that for each increase of 1 eq. of bicarbonate in the plasma about 0.75 eq. of chloride enters the cells. This ratio is approximately that for mammalian blood (Van Slyke, 1921). In trout blood, however, the loss of chloride from the plasma at the maximum cell volume greatly exceeds the increase in CO_2 in the plasma. This suggests that the excessive cell volumes and excessive chloride shift may be due to the production in the blood of an acid other than CO_2. It is immaterial where this acid is produced, but it is necessary to postulate that it is diffusible through the red cell membrane. It is also necessary to postulate that it is produced by a reversible reaction and that the equilibrium point is determined by the tension of oxygen and of CO_2 in the blood. The optimum conditions for its formation would presumably be at low oxygen tensions and a CO_2 tension between 10 and 20 mm. To explain the cell volume changes by

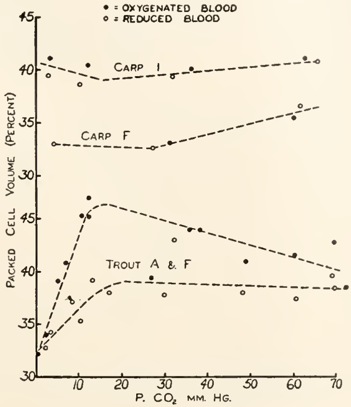

FIG. 3. Packed cell volumes in carp and trout blood are plotted against the pressure of CO_2. The large changes in cell volume in trout blood are in striking contrast to the small changes in carp blood.

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this production of acid alone would require the formation of about 30 m.eq. per liter of acid other than CO_2. Such an hypothesis certainly deserves the utmost skepticism till the changes in cell volume can be checked by a method other than centrifuging, but it does receive some support of a qualitative nature from another and independent consideration, namely the effect of oxygenation on CO_2 capacity.

Oxygenation and CO_2 Capacity

When the effect of oxygenation on CO_2 capacity is expressed as $-\Delta BHCO_3/\Delta O_2$ at constant plasma pH the maximum values for this ratio are found at a pH of about 7.3 in both bloods. The maximum value in each is about 1.2. This value is higher than any of those in other species compiled by Redfield (1933), as it should be, in accordance with the principle that the greater the effect of acid on the combination of oxygen the greater should be the effect of oxygenation on the dissociation of hemoglobin as an acid. But the fact that the value is the same in trout and carp blood is apparently inconsistent with the principle. If, however, the hypothesis of " extra acid " production in trout

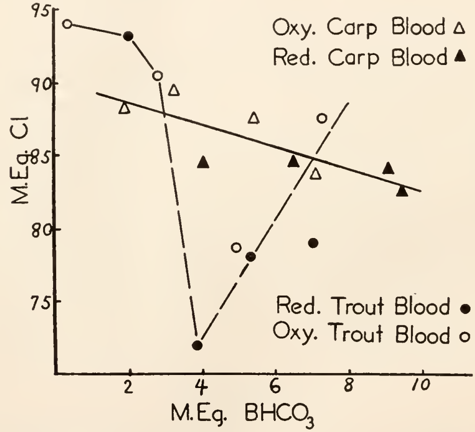

FIG. 4. The chloride in the plasma of a liter of blood is plotted against the bicarbonate in the same volume to illustrate the magnitude of the migration of chloride into the cells. In carp blood $\Delta BHCO_3/\Delta Cl = -.75$ as in mammalian blood, but in trout blood the ratio is much greater than unity, suggesting that an acid other than CO_2 has been neutralized in the cells and diffused into the plasma in exchange for chloride ions.

blood is correct, the apparent inconsistency would be only apparent, for if an acid other than CO_2 were produced in the blood at low pressures of oxygen, $\Delta BHCO_3$ would not be a complete measure of the change in base combined with hemoglobin, and hence would give too low an estimate of the effect of oxygenation on the acid dissociation of the hemoglobin in trout blood.

Buffer Power

The buffer power (β) of whole blood is often represented by the ratio $-\Delta BHCO_3/\Delta pH_s$, where $\Delta BHCO_3$ is the change in CO_2 combined in whole blood and pH_s the pH of the plasma or serum. This procedure will be erroneous in trout blood if the hypothesis of the extra acid production is correct. It is interesting, however, to make the calculation in the conventional manner and then to consider what change in conclusion would be necessary if the hypothesis of extra acid production were correct. The calculated buffer powers of carp, trout and human blood adjusted to equal concentrations of hemoglobin are given in Table IV. Figures are given for reduced blood only, since fully oxygenated trout blood cannot be obtained over a large enough range of pH. Carp blood has about the same buffer power over the range studied, but trout blood shows a greater buffer power over the more physiological range of P CO_2 of 2–20 mm., where it is equal to the buffer power of human hemoglobin. Over the higher range of CO_2 pressure the buffer power of trout hemoglobin is less, though still higher than that of carp blood. The trout blood shows a concentration of its buffer power in the physiological range, a characteristic which may be attributed tentatively to closer grouping of the dissociation constants of

TABLE IV

Buffer power of reduced bloods.

	Range of plasma pH	Range of P CO_2	Buffer power (β)
Carp 15°	8.11–7.36	2–20	1.6
	7.36–6.96	20–60	1.4
Trout 15°	7.90–7.23	2–20	2.3
	7.23–6.85	20–60	1.7
*Human 38°	7.5–7.2	35–90	2.3

* Peters and Van Slyke, Quantitative Clinical Chemistry, Vol. I.

$\beta = -\Delta BHCO_3/\Delta pH/Hb$. Concentrations are in milliequivalents per liter.

the acid (or basic) groups in this range. If the anomalous increase and decrease in cell volume in trout blood represents increase and decrease in extra acid, the true buffer power over the range 2–20 mm. would be even greater, and even less over the range 20–60 mm. In other words, the tendency for the buffer power to be concentrated in a narrow range in the trout blood would be even more striking.

Plasma pH

Values of plasma pH at CO_2 pressures of 2, 20 and 60 mm. Hg calculated from the smoothed data of the dissociation curves, assuming a pK_1' of 6.2 are shown in Table V. The changes in plasma pH may be taken as paralleling fairly closely the changes in cellular pH. To calculate these separately would merely introduce the uncertainties of arbitrary values for pK_1' in cells.

The main points of interest are: (1) an increase of P CO_2 from 2 mm. to 20 mm. produces a greater change in pH in carp blood than in trout blood; (2) at P CO_2 of 60 mm. the carp blood is as acid as the trout blood. In other words, the loss in oxygen capacity in trout blood cannot be attributed either to a greater change in acidity for a given increase in P CO_2 or even to a higher absolute acidity at the higher pressures of CO_2. However, the lower acidity in carp blood at lower pressures of CO_2 must be held partly responsible for the higher affinity of the blood for oxygen in the absence of CO_2.

TABLE V

pH of true plasma of oxygenated blood calculated from the data of Tables II and III.

P CO_2	Carp	Trout
mm. Hg		
2	7.91	7.66
20	7.23	7.15
60	6.84	6.84

DISCUSSION

The results as a whole indicate that the great differences in the effect of CO_2 on the combination of oxygen in these two bloods and their affinity for oxygen may be due to adaptations at three levels of physiological organization. These seem to be: (1) specific differences in the hemoglobin molecule; (2) differences in the environment provided for the hemoglobin by the erythrocyte; (3) differences in the acid-base regulation of the fish as a whole. It seems likely that differences in the hemoglobin molecules will prove to be the most important element in the total adaptation, although no comparison of the two hemoglobins in solution has yet been made. Until such a comparison is made, it cannot be said that differences in the erythrocytes may not be equally important. Certainly one of the most striking differences between the bloods has been the behavior of the cell volumes. The effect of the intracellular environment on the affinity for oxygen is marked, even among mammals, and varies from species to species (Hill and Wolwekamp, 1936) in a manner as yet inexplicable. In fish blood the effect of hemolysis on affinity for oxygen is also large and cannot be explained by changes in acidity alone (Root and Irving, 1940). The anomalous behavior in trout blood of cell volumes, plasma chlorides and effect of oxygenation on CO_2 capacity could all be explained, at least in part, by the production (by a kind of Pasteur reaction) of acid at low tensions of oxygen and an optimal tension of CO_2. The production of extra acid cannot, however, explain the loss of oxygen capacity or the convergence of the CO_2 dissociation curves of oxygenated and reduced blood. At most it could only be a mechanism augmenting the effect of CO_2 in maintaining a high tension of oxygen at low contents of oxygen in the blood of trout.

SUMMARY

The venous blood from four species of freshwater fish, rainbow trout (*Salmo gairdnerii* Richardson), carp (*Cyprinus carpio* Linnaeus), bullhead (*Ameiurus nebulosus* Le Sueur) and sucker (*Catostomus commersonii* Lacepède) was analyzed and an estimate made of the probable range of physiological CO_2 tension.

A detailed study was made of CO_2 transport in the blood of two of these species, the rainbow trout and the carp, which differ greatly in the effect of CO_2 on the combination of oxygen in the blood. They differ too, in their systems of CO_2 transport. A curious feature of trout blood is the great change in packed cell volume with changes in the pressure of O_2 and CO_2. An hypothesis is presented to explain in part these anomalous changes in cell volume and other characteristics of the trout blood. Carp blood shows less differentiation from general vertebrate characteristics.

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THE DEVELOPMENT OF THE ASCIDIAN EGG CENTRIFUGED BEFORE FERTILIZATION

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The development of centrifuged eggs of ascidians has been studied by Duesberg (1926) and by Conklin (1931). In *Styela* and *Ciona* Conklin found that when the eggs were centrifuged after fertilization, three substances, namely the mitochondria, the hyaloplasm, and the yolk may be displaced from their normal positions into three zones. If these substances were so held until the beginning of cleavage, they were distributed abnormally to the blastomeres. The tissues or organs of the embryos derived from these eggs were also dislocated.

The unfertilized eggs of *Styela* and *Ciona* also were centrifuged by Conklin. He noted that under strong centrifuging the spermatozoön frequently did not enter the egg at all. No description of the development of these eggs was given.

Recently Dalcq (1932, 1935, 1938) has fragmented unfertilized eggs of *Ascidiella scabra* into two parts and then fertilized them. He found that meridional halves of egg fragments may give rise either to apparently normal and symmetrical larvae, or to lateral half larvae; while larvae obtained from latitudinal halves may be deficient in one or more kinds of tissues according to the level of the cut. These experiments indicate that the unfertilized egg already possesses germinal localizations; but at the same time it has a great capacity for regulation, as has been demonstrated by Tung (1934) for the fertilized egg.

To gain further light on the organization of the unfertilized egg of the ascidian and to learn whether, with sufficient force, the organ-forming substances might be dislocated, we have made a study of the development of *Ciona* eggs strongly centrifuged before fertilization. The experiments on which this investigation is based were performed in the summer and autumn of 1936.

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[1] This work was done while the senior author was on the tenure of a grant from the Board of Trustees for the administration of the indemnity funds remitted by the British Government for which he wishes to express his gratitude.

MATERIAL AND METHODS

The observations recorded here were all made on eggs of *Ciona intestinalis* obtained in the vicinity of Tsingtao. During the months from June to October the gonoducts of *Ciona* are usually full of ripe eggs and spermatozoa. The eggs were obtained free from sperm by removing the integuments and carefully opening the oviduct which is near the surface. The eggs were then removed from the oviduct with a pipette and transferred to a glass dish containing fresh sea water. A part of the eggs was used for centrifuging, leaving the remaining eggs in the dish as a control.

The eggs to be centrifuged were placed in glass tubes with 10 cc. sea water and rotated for ten minutes to one hour and fifty minutes at a speed ranging from 2000 r.p.m. to 3800 r.p.m. These speeds represented centrifugal forces of about 716 × gravity and 2585 × gravity. In order to prevent eggs from rotating during centrifugation, capillary tubes were used as by Conklin. After centrifuging, the eggs were immediately removed from the capillary tubes and fertilized by sperm of another animal.

Individual eggs showing an abnormal distribution of oöplasmic substances to the first two or four blastomeres were picked out, sketched, and placed in separate dishes of fresh sea water in order to study in detail the location of various tissues in later development.

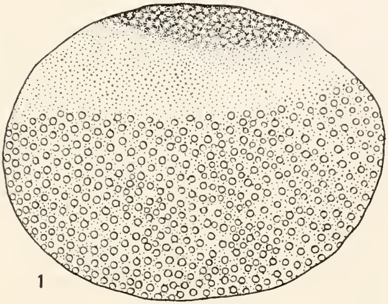
The larger part of the eggs and embryos was fixed in Bouin's fluid and double-embedded in agar-paraffin (Chatton, 1927). Sections were cut at 7 μ and stained with iron-haematoxylin, eosin, and light green. Some of the material was fixed in Flemming's solution and mounted in toto in order to determine the location of mitochondria.

RESULTS

Stratification of the Egg

The degree of the stratification of oöplasmic substances of the unfertilized egg varies with the rate and duration of centrifugation. In strongly centrifuged eggs three zones can be distinguished. These are: (1) an alveolar or light zone at the centripetal pole; (2) a middle clear zone containing the nuclear elements; and (3) a lower, heavy zone of yolk. In eggs fixed in Flemming's solution and mounted in toto, the light zone consists exclusively of large black, densely packed granules. On the basis of the staining reaction, it corresponds obviously to the "mitochondria" zone of Duesberg. Sections of the eggs show that this zone is composed of an alveolar substance in which are embedded a large number of blue-black granules; these are taken to be mitochondria (Fig. 1).

Under prolonged centrifuging, a fourth zone appears at the centrifugal pole. Figure 2 shows an egg in which the four zones are clearly separated. The centrifugal zone contains blue-black granules, while in the centripetal zone the number of the same kind of granules is greatly reduced. Figure 3 shows an egg with incomplete stratification of the fourth zone. In this case a part of the granules remains



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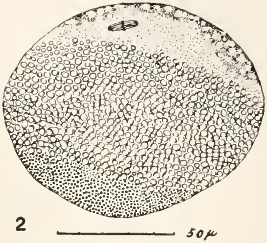

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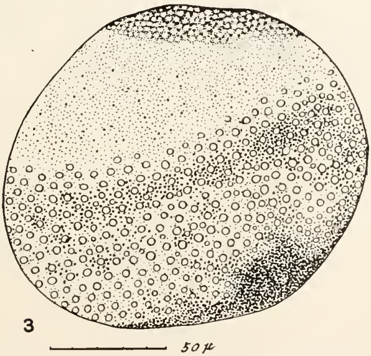

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Fig. 1. Section of an unfertilized egg, centrifuged for one hour at 2193 times gravity, and fixed ten minutes later, showing a stratification of the egg contents into three zones.

Fig. 2. Section of an unfertilized egg centrifuged for one hour at 2193 times gravity and fixed ten minutes later, showing the fourth zone at the centrifugal pole.

Fig. 3. Section of an unfertilized egg, centrifuged for one hour at 2193 times gravity and fixed fifteen minutes later. The stratification of the centrifugal zone is not complete.

scattered throughout the clear and yolk zones and especially in the latter. A comparison of the eggs illustrated in Figs. 1, 2, and 3 leaves no doubt that the granules of the centrifugal zone are separated out from the alveolar substance of the centripetal zone in which they were formerly embedded.

Conklin described a fourth zone between the hyaline and yolk zones and concluded that ". . . It (the substance of the zone), rather than the mitochondria, is the myoplasm or formative substance for the future muscles." Our conclusions are that it is the alveolar substance of the centripetal zone that is the formative substance of the muscles; the alveolar substance is similar to the cytoplasm of muscle cells of the young embryo both in structure and in staining reaction, whereas the granules of the centrifugal zone are the mitochondria since in normal fertilized eggs similar granules are found in the lower hemisphere at which the sperm enters. In *Physa heterostropha,* Clement (1938) has recently shown that the mitochondria which stratify between the clear and yellow zones and are therefore heavier than the clear protoplasm are the last to be segregated under centrifugal forces. In these respects our observations on *Ciona* agree with those of Clement on *Physa.*

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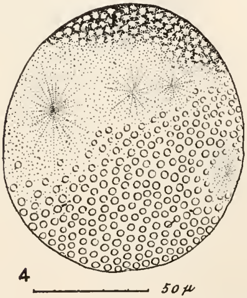

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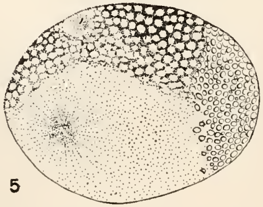


FIG. 4. Section of a polyspermic egg centrifuged for one hour at 2193 times gravity and fixed fourteen minutes after fertilization. Note sperm asters in clear and yolk zones.

FIG. 5. Section of a polyspermic egg centrifuged for one hour at 2193 times gravity and fixed fourteen minutes after fertilization. Note sperm asters in clear and alveolar zones.

Immediately after centrifuging, the eggs were cross-fertilized with fresh sperm. As a check, in each experiment, the control eggs left unfertilized in the original dish were examined. As has recently been reported by Morgan (1938), cleavages were rare, showing that self-fertilization occurs rarely in *Ciona.* Conklin found that in eggs which had been strongly centrifuged before fertilization, the spermatozoön frequently did not enter at all. He attributed the failure of fertilization to the compactness of the yolk at the vegetative pole which blocked the entrance of the spermatozoön. In our experiments, though we have no detailed record, the percentage of fertilization in centrifuged eggs is generally not very low in comparison with normally fertilized eggs.

In sections it appears that the spermatozoön may penetrate the egg in the clear zone or between this zone and the yolk; for the most part it enters the yolk zone (Fig. 8). This indicates that the compactness of the yolk is not a factor in blocking the entrance of the spermatozoön. In polyspermic eggs the sperm asters are found in almost any part of the egg, even in the alveolar substance of the centripetal zone. Figures 4 and 5 show two such eggs that had been centrifuged for one hour at 2193 × gr. and were fixed fourteen minutes after fertilization. The entrance points of the sperm are suggested by the positions of the asters.

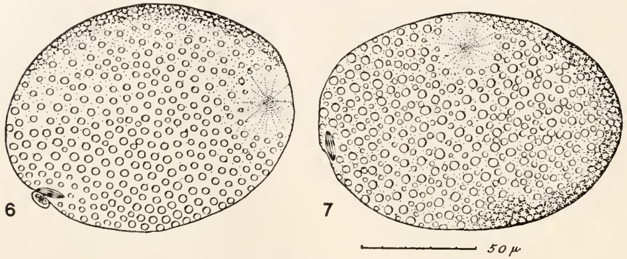
The first maturation spindle of the strongly centrifuged eggs always lies in the clear zone. After fertilization it moves to the periphery of the zone where the polar bodies are given off. In some polyspermic eggs, when a sperm-nucleus has already migrated to the center of the egg, the first maturation spindle still rests in the middle of the clear zone. In such cases probably no polar bodies will be given off; they will be retained in the egg.

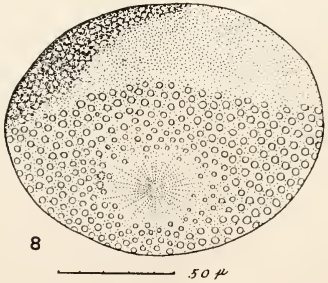
The entrance point of the spermatozoön may be found at any plane with respect to the position of polar bodies. This is evident in Figs. 6 and 7, which represent two eggs centrifuged for ten minutes and fixed one hour after fertilization.

In the normal egg a protoplasmic movement usually takes place immediately after the entrance of the sperm. Such movements probably also occur in the weakly centrifuged eggs with incomplete stratification of oöplasmic substances, since after fertilization the different substances of such eggs return to their normal positions. In strongly centrifuged eggs the movement of the oöplasm is less marked. This is well demonstrated in Fig. 8. This egg was centrifuged in a capillary tube for one hour and twenty-four minutes at 2193 × gr., and fixed forty minutes after fertilization. The three principal zones are still quite clear. The sperm aster is found in the yolk zone surrounded by a clear substance and no mitochondria. It appears that the free movement of the substances is impeded by the stratification.

Cleavage

As a check, in every experiment some of the control eggs were fertilized at the same time as the centrifuged eggs. The first cleavage of the eggs of both sets occurred at the same time, regardless of the rate and duration of centrifuging. It took place usually in about one hour and a half after insemination, much later than the records of other authors. Such delayed cleavage may be due to the low temperature of the sea water at Tsingtao.

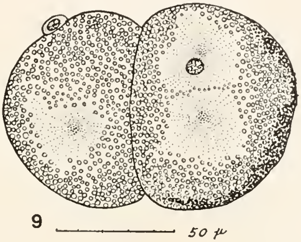




Figs. 6 and 7. Sections of eggs centrifuged for ten minutes at 2193 times gravity and fixed one hour after fertilization, showing the positions of the sperm aster, the polar body (Fig. 6) or second maturation spindle and the mitochondria crescent. There are no constant relations between them.

Fig. 8. Section of an egg centrifuged in capillary tube for one hour and twenty-four minutes and fixed forty minutes after fertilization. The sperm aster is in the yolk zone; no mitochondria surround it.

The pattern of the cleavage planes of the centrifuged eggs differed in no essential respect from those of normal eggs. The first two cleavage planes were perpendicular to each other, while the third plane was at right angles to both of the first two, resulting in the formation of four micromeres and four macromeres. However, the first two cleavage planes did not always pass through the point of attachment of the polar bodies. After prolonged centrifuging, there was a significant percentage of eggs whose polar bodies were not at the first cleavage furrow. The deviation between them may be of any angle up to 90° (Fig. 9). Since after centrifuging there was no definite landmark to indicate the original position of the animal pole and since the orientation of the eggs during the process of centrifuging could not be determined accurately, it was impossible to ascertain whether the polar bodies were produced at other than their normal position, or whether the first cleavage plane passed through the original animal pole.



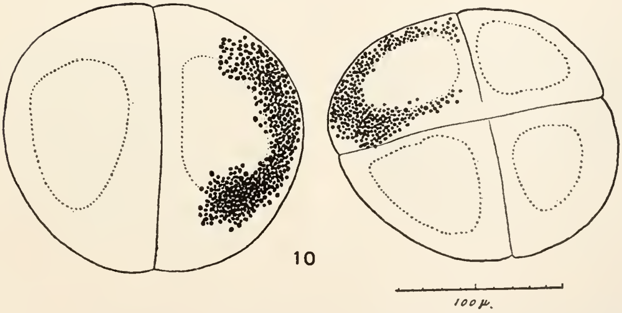
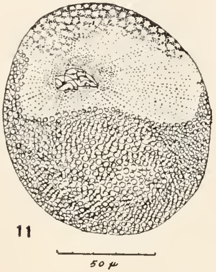
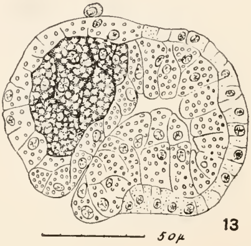


Fig. 9. Section of an egg in 2-cell stage. The polar body is not located at the cleavage furrow.

Fig. 10. Two entire eggs in 2- and 4-cell stages, showing mitochondria in one blastomere.

In most eggs the first cleavage plane appears to coincide with the axis of centrifugation. As the stratified substances are rarely equally distributed around the axis, it divided them into more or less unequal halves. However, there is a significant percentage of eggs in which the first cleavage appeared in any plane and at any angle with respect to the stratification of substances. It may be oblique to the axis of centrifugation or perpendicular to it. In the latter case one blastomere contains only the yolk spherules and the other the clear and alveolar substances. Figure 10 shows two such eggs in the 2- and 4-cell stages respectively. They were fixed in Flemming's solution and mounted in toto. The mitochondria or alveolar substance is confined to one blastomere. Similar conditions are also shown in Fig. 9.





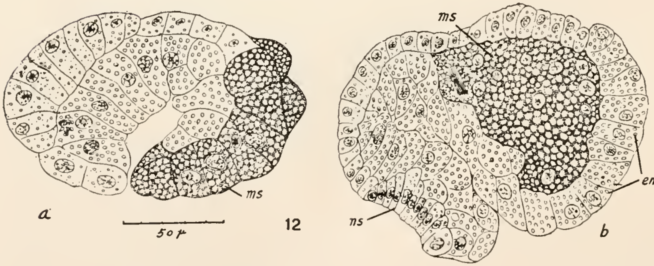


FIG. 11. Section of an egg centrifuged for one hour at 2193 times gravity. The three oöplasmic zones remain unchanged after fertilization.

FIG. 12. Section of two gastrulae, showing the abnormal positions of muscle (*a*) and endoderm (*b*) cells. *ms.*, muscle cell; *end.*, endoderm; *ns.*, neural cell.

FIG. 13. Section of a gastrula derived from egg centrifuged fifteen minutes at 2193 times gravity. The polar body is situated posterior to the middle of the ventral surface in its typical position.

It not infrequently happens that the first cleavage is quantitatively unequal; this leads to a formation of a larger macromere and a smaller micromere. Sometimes the second cleavage plane does not intersect the first at right angles, so that the four resulting blastomeres do not lie in the same plane. The cleavage pattern in such eggs as well as in those described above seems to be determined by the position of the cleavage spindle. In cases where the three principal zones remain unchanged after fertilization, the first cleavage spindle always lies horizontally in the clear zone. In such eggs the cleavage plane coincides with the axis of centrifuging. If a slight change of the stratification takes place before the division, the position of the mitotic spindle is also changed. The subsequent cleavage will then divide the egg in any plane with respect to the axis of the centrifuging. Figure 11 shows a section of an egg in which the oöplasmic substances were stratified into three zones. The two pronuclei had come together in the center of the clear zone. The first cleavage of this egg may be expected to approximate the axis of stratification and will distribute the oöplasmic substances equally between the two blastomeres.

In many eggs the division of the cell body is suppressed while the division of centers and chromosomes continues. In such eggs are found numerous nuclei and centers, confined to the alveolar and clear zones. Such anomaly of cleavage is evidently not due to any direct effect upon the mitotic figure, for the fertilization of the eggs took place after centrifuging.

Later Development of Centrifuged Eggs

The later development of centrifuged eggs is particularly interesting since the dislocated tissues or organs can be identified in stained sections. Individual eggs showing abnormal distribution of oöplasmic substances to the first two, four, or eight blastomeres were sketched and isolated. The development of these eggs was studied. In general, it may be said that the stronger and the longer the centrifuging, the more abnormal was the subsequent development.

The gastrulation of strongly centrifuged eggs was rarely typical. Figure 12 shows two abnormal cases in which a part of the mesoderm and entoderm cells had not been invaginated. In normal development the polar bodies are situated on the anterio-ventral part of the older gastrula. In Fig. 13 a polar body is found posterio-ventrally on the gastrula, indicating that gastrulation took place independent of the position of the polar bodies.

Following prolonged centrifugation, a large number of eggs started their development but most of them never reached a stage at which they could be recognized as larvae. The embryos were so abnormal in form that it was impossible to identify their parts and organs except by a histological study. In these embryos, tissues and organs which will be described separately in the following paragraphs were usually displaced. Embryos are found composed of three types of cells, namely: (1) muscle and mesenchyme cells; (2) ectoderm and neural cells; and (3) notochordal and endoderm cells. These types correspond to the products of the three zones (viz., alveolar substance, clear cytoplasm, and yolk) which had been stratified by the centrifugal force.

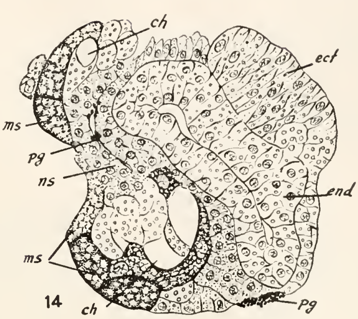
Muscle Cells and Mitochondria.—In typical embryos and larvae, the large muscle cells are arranged in the tail in three rows on each side of the notochord. Owing to their large nuclei and specific staining reaction, they are easily distinguished from other types of cells. In the abnormal larvae derived from centrifuged eggs, isolated or aggregated cells of this type may be found in the interoir or at the surface (Fig. 14). They are rarely arranged regularly even when they are found alongside of the notochord. In some cases these cells lie in the midst of the endoderm or just under the neural tissue. Figure 15 shows a section of a larva in which a part of the gut wall is formed of cells other than typical endoderm. Though one cannot be certain as to whether these are really mesoderm cells or not, the structure of the cytoplasm and the size of the nucleus are similar to those of the latter.

The mitochondria which are normally embedded in the cytoplasm of the muscle cells may have been driven into the regions subsequently forming ectoderm (Figs. 16 and 19*a*) or neural tissue (Fig. 15). The myofibrillae are often found in the muscle cells, but are never found in those tissues into which the mitochondria have been driven. Our observations, therefore, confirm the view of Conklin, that in *Ciona* the mitochondria do not give rise directly to myofibrillae.

Notochordal Cells.—Owing to the abundance of yolk spherules, the notochordal cells of the young embryo are very similar to those of the endoderm. In larvae, on the other hand, they are characterized by the possession of large vacuoles and can be thus recognized wherever they occur. In most abnormal larvae derived from strongly centrifuged eggs, notochordal cells did not arrange themselves to form a rod, but instead were displaced to various positions. They may be grouped together or scattered. In some cases, they are found at the surface (Fig. 17) of the larvae or just under the ectoderm (Fig. 16). In other cases they are in the midst of endoderm or surrounded by muscle cells. In such embryos no tail is formed.

The neural tissue which will be described in the next paragraph was not always associated with the notochordal cells; nor do the latter exert any influence on the differentiation of the ectoderm cells with which they are in contact. We agree, therefore, with Berrill and Conklin who have concluded that the notochord of the ascidian does not act as an organizer in the sense of Spemann.

Neural Cells and the Sensory Pigment.—The neural cells may occur in any portion of these abnormal larvae. Sometimes they formed a



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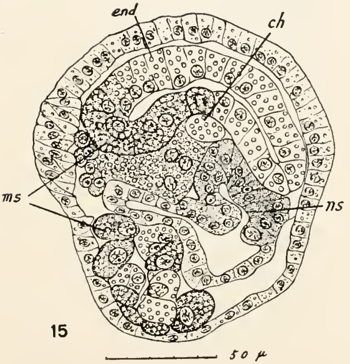

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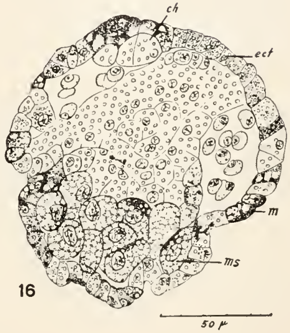

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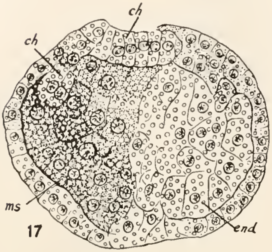
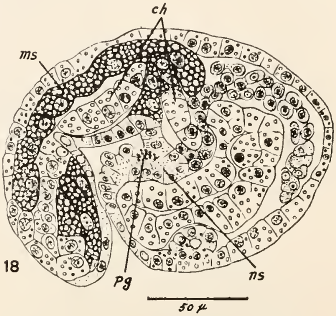
FIG. 14. Section of an abnormal larva derived from an egg centrifuged for thirty minutes at 2193 times gravity and fixed twenty-eight hours after fertilization. *ms.*, muscle cell; *ch.*, notochord; *pg.*, pigment spot; *ns.*, neural cell; *ect.*, ectoderm; *end.*, endoderm.

FIG. 15. Section of an abnormal larva derived from an egg centrifuged for one hour and thirty minutes at 2193 times gravity and fixed twenty-four hours after fertilization. A part of the gut wall contains alveolar substance. *ms.*, muscle cell; *ns.*, neural cell; *end.*, endoderm; *ch.*, notochord.

FIG. 16. Section of a larva from an egg centrifuged for one hour and fifty minutes, showing the mitochondria in the ectoderm and neural cells. *m.*, mitochondria; *ms.*, muscle cell; *ch.*, notochord.

plate on the surface; at other times they were grouped forming a mass of neural tissue. Usually, however, they lined a large irregular cavity extending into the interior of the larva. In larvae having well-developed tails, such a neural cavity is always found at the junction of the trunk and the tail. The latter often turned dorsally, giving the larvae a curvature in an atypical direction. Figure 18 shows a larva of this kind. The neural cavity with two spots of sensory pigment is surrounded by the curved tail on one side. Conklin has suggested that the elongation of the neural plate and tube depends upon the normal elongation of the notochord. Our observations, however, do not lead to this conclusion.





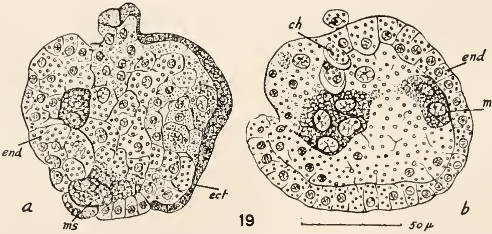


Fig. 17. Section of a larva from an egg centrifuged in capillary tube for forty minutes at 2193 times gravity. *ch.*, notochord; *ms.*, muscle cell; *end.*, endoderm.

Fig. 18. Sagittal section of a larva derived from an egg centrifuged one hour and thirty minutes at 2193 times gravity, showing the inverse body curvature. *ch.*, notochord; *ns.*, neural tissue; *pg.*, pigment spot; *ms.*, muscle cell.

Fig. 19. *a.* Section of a larva from an egg centrifuged in a capillary tube for fifty minutes at 2585 times gravity. The left yolk-filled cells are endoderm and the right mitochondria-filled cells are ectoderm.

b. Section of a larva from an egg centrifuged for fifteen minutes at 2193 times gravity. The yolk-filled cells form a superficial epithelium. *ch.*, notochord; *ect.*, ectoderm; *end.*, endoderm; *ms.*, muscle cell.

The sense organs are also structures of interest. In typical larvae there are two sense organs, the eye and the otocyst, with their pigments situated on the wall of the brain vesicle. Typical organs are rarely formed in abnormal larvae; instead, pigment spots are found in unexpected places. The number of spots varies from none to four. Their locations may be near together or widely separated (Fig. 14). In some cases they project from the surface of the larva and in others they are embedded in the neural cells. However, they are always associated with neural tissue and notochordal cells.

Endoderm and Ectoderm Cells.—The histological characteristic of endoderm is its large yolk-filled cells. In these abnormal embryos, however, it is not easily identified, for the yolk spherules are also found in other cells of the embryo. In such a case as that shown in Fig. 19*A* there is no doubt but that the superficial cells of the left side are endodermal and those of the right side are ectodermal. In the case shown in Fig. 19*B*, on the other hand, one cannot be certain as to whether the yolk-filled surface epithelium labeled " end " is true endoderm or is ectoderm. Tung (1934), in experiments on the combination of blastomeres in *Ascidiella scabra,* has shown that the development of ectoderm and endoderm is not strictly mosaic. When the endodermal cells lie at the surface of the embryo, they may form a regular superficial epithelium. Similarly, if ectodermal cells come to lie in the interior, they may transform into large irregular endoderm-like cells. Such a regulative capacity of ectoderm and endoderm seems to exist also in the tissues of *Ciona* embryos.

In these larvae having displaced organs, the endodermal cells may be found in many abnormal locations. The fact that they are not always associated with neural tissue and pigmented sensory spots indicates that they do not serve as organizers.

Papillae.—The normal embryo possesses three papillae; two are paired and are situated dorso-laterally on each side; one is median and ventral. Papillae are also observed in some of the abnormal larvae. Their number varies from none to three. It is of interest to note that in spite of the dislocation of other organs, the development of papillae is always in relation to the endoderm. Without exception in all 49 cases observed, the papillae were formed in ectoderm underlain by endoderm. Tung (1934), on the basis of experiments on *Ascidiella scabra* in which the four animal blastomeres were rotated through 180° over the four vegetal blastomeres, has suggested that papillae are evoked in the ectoderm by underlying endoderm. The present observation furnishes further evidence in support of that hypothesis.

From the above descriptions it is obvious that the different tissues and organs of the many larvae derived from eggs centrifuged before fertilization, are out of their normal positions. Such placement of tissues is undoubtedly the result of the placement of the oöplasmic substances from which the tissues are derived. In some cases the quantity of certain tissues seems to be reduced. We have not, however, made a count of the cellular components of these tissues.

DISCUSSION

Conklin (1905 *a* and *b*), in his classical studies on the development of ascidians, described four different kinds of oöplasmic substances in the fertilized egg, namely the ectoplasm, endoplasm, mesoplasm, and chorda-neuroplasm. From these are derived the ectoderm, endoderm, mesoderm, and the notochordal and neural tissue respectively. According to Conklin (1905*c*), the development of these tissues or organs is strictly mosaic. When the eggs were centrifuged after fertilization, tissues of the larvae which developed were displaced from their normal positions (1931). Such displacements of tissues were attributed to corresponding displacements of oöplasmic substance by means of centrifugal force.

As has been shown in the present experiments, the stratification of oöplasmic substances of eggs centrifuged prior to fertilization and the displacement of the tissues or organs of the larvae derived from these eggs are in general similar to those obtained from eggs centrifuged after fertilization. It is reasonable, therefore, to conclude that the different kinds of oöplasmic substance described by Conklin in fertilized eggs have already existed in the egg before fertilization. This conclusion agrees with that of Dalcq (1932, 1935, 1938), who likewise concluded that organ-forming substances are already differentiated in the unfertilized eggs. Conklin has suggested that the unfertilized egg of *Cynthia* possesses a bilateral symmetry. If so, it is undoubtedly due to the bilateral arrangement of these oöplasmic substances.

In recent years the idea that the ascidian egg is strictly mosaic has been called in question, though it is still insisted upon by Berrill (1932) and by Cohen and Berrill (1936). Schmidt (1931) observed three papillae in larvae derived from one-half blastomeres of *Ciona* and *Phylusia* eggs. Reverberi (1931) has obtained normal larvae from fragments of fertilized *Ciona* eggs. Tung (1934) has found in *Ascidiella scabra* that the endoderm and ectoderm are relatively equipotential and that the development of papillae and sensory pigments is always associated with endoderm and notochord respectively. Recently, Von Ubisch (1938) has described a normal embryo of *Ascidiella aspersa* produced by fused eggs. All these investigations show that after fertilization the ascidian egg has a considerable capacity for regulation.

Dalcq has reported a certain degree of regulation in the unfertilized egg of *Ascidiella scabra.* A fragment of the egg may give rise to an embryo very similar to the control. The number of cells of muscles or notochord of a pair of embryos derived from two fragments of one egg may be double the total number of a normal embryo. Moreover, in the experiments reported here, we have demonstrated that the ectoderm and endoderm constitute relatively equipotential systems and that the papillae and sensory pigment do not appear to be self-differentiating organs. These facts plainly show that the eggs of *Ascidiella* and *Ciona* are not strictly mosaic prior to fertilization.

The recent work of Rose (1939) shows that the anterior vegetal region of the *Styela* egg is the cerebral inductor. This region contains materials essential to the differentiation of endoderm, notochord and a part of the neural tissue. In the larvae with displaced tissues or organs we have not found any typical relations between the cerebral vesicle and either notochord or endoderm. In short, there is no evidence in the present experiments to indicate that either notochord or endoderm acts as a cerebral inductor. The development of the adhesive papillae has been discussed by Cohen and Berrill (1936). They have found three papillae in a lateral half larva of *Ascidiella aspersa* and interpret the origin of such supernumerary papillae as a result of the mosaic pattern. If this interpretation is correct, it might be expected that in larvae with displaced organs, papillae would be found in a variety of abnormal locations. On the contrary, papillae in abnormal larvae are always associated with the endoderm. This fact furnishes further evidence in favor of the suggestion of Tung that papillae appear to be evoked in the ectoderm by the underlying endoderm.

From the foregoing discussion we come to the conclusion that the organization of the unfertilized egg of the ascidian is similar to that of the fertilized egg. In both there exist different kinds of oöplasmic substances from which different tissues develop. These substances, however, are not strictly mosaic; they still possess a certain capacity for regulation. Some organs, such as papillae and sensory cells, seem incapable of self-differentiation. Their development might be dependent upon extrinsic factors.

Summary and Conclusions

1. The unfertilized eggs of *Ciona intestinalis* after centrifuging can be cross-fertilized. The majority undergo cleavage but rarely develop normally.

2. The first cleavage furrow may lie in any plane relative to the position of polar bodies or the axis of centrifuging.

3. In larvae derived from strongly centrifuged eggs, tissues and organs were often displaced from their normal positions.

4. Endoderm and ectoderm appear to be relatively equipotential.

5. There is no evidence that the differentiation of the neural tissue is dependent upon other tissues with which it is in contact.

6. Mitochondria may be displaced from muscle cells and appear in

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the neural or ectodermal cells, where they are not transformed into myofibrillae.

7. The number of papillae developed by the abnormal larvae varied from none to 3. They are always adjacent to the endoderm which it is suggested may evoke their formation in the ectoderm.

8. The number of sensory pigment spots in the abnormal larvae varies from none to 4. They are always associated with notochordal cells.

9. The organization of unfertilized eggs is found to be strikingly similar to that of fertilized eggs in respect to oöplasmic substances and the capacity of regulation.

10. The elongation of the notochord is not always accompanied by the elongation of the neural plate or tube.

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THE DEVELOPMENT OF THE BUD IN BOTRYLLUS

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In the following account an attempt is made to describe the development of the bud in *Botryllus* in a manner directly comparable with the development of an egg, in an effort to bring out certain essential simplicities in this direct type of development. The subject itself is not new, but it is believed that the treatment is. Of the older papers those of Hjort (1896) and Pizon (1893) are outstanding. Hjort's accounts were concerned with the significance of the primary germ layers, or rather their lack of significance, in the asexual development of *Botryllus.* Pizon, on the other hand, described primarily the formation of young colonies rather than bud development as such. Also his work was marred by a completely erroneous account of the origin of the gonads, gonads being considered to arise in the oozoöid, in conformity with the opinion of Weismann, and to be transported to the developing buds of succeeding generations until sexual maturity was finally attained. This was a false conception, and the description given here is very different.

Origin and Nature of Bud

The bud of *Botryllus* first appears as a small disc-like thickening of the atrial epithelium on each side of the body, immediately anterior to the gonads. A bud appears on each side, while just posterior to them hermaphrodite gonads continue to develop. The atrial epithelium is of ectodermal origin, and apart from its involvement in the process of budding plays its part apparently only as a limiting membrane. There is, in other words, no reason to suppose that the cells of the atrial epithelium have become in any way specialized. Their formation as an epithelium is a matter of tissue organization and implies nothing in regard to cell specialization.

The disc of atrial epithelium concerned in budding is overlain externally by a similar area of epidermis. This tissue is likewise an epithelium and of ectodermal origin, but its association with test or tunicin production suggests that it has special chemical activities in addition to serving as a limiting membrane. That is, its constituent cells have probably acquired a certain degree of individual specialization.

The double disc of cells forming the initial bud is shown in Fig. 1, in optical section. In Fig. 1, *A* it is shown in relation to its subsequent developmental cycle inasmuch as three stages are shown while yet in organic continuity. In the largest and oldest of the three generations, viewed from the ventro-posterior side, the vascular connection with the circulatory system of the colony is clearly seen. The zoöid is fully active and the contained eggs have developed as far as the gastrula stage. The zoöid bears on its right side a bud about one-fifth its own length in which the organization is virtually completely expressed but is far from being functional. This bud has also formed vascular connection with the colonial system. In turn it bears a bud in the first or disc stage. Two features may be emphasized. A high degree of structural organization is attained at a relatively small size, and the size of the bud in the disc stage is minute when compared both with its size at the end of development and also with that of the developing egg. In Fig. 1, *B* the bud disc is shown, on a larger scale, in relation to the adjacent structures of the parent bud.

Polarity

The question of origin of polarity in ascidian buds has been discussed before (Berrill, 1935, 1936). In every case where organic continuity is maintained between bud and parent, and *Botryllus* is no exception, the polarity of the bud is clearly a derivative of that of the parent. Both the antero-posterior axis and the left-right axis coincide with those of the parent zoöid and must exist from the beginning.

Development of the Bud as a Whole

The simplest conception of the developing bud is that of a mass of tissue expanding during a certain period. This is shown pictorially in Fig. 2. The larger drawing in this figure represents accurately the linear growth plotted against time. The various cross-sections within the cone of growth represent certain developmental stages of special interest. At 26° C. the time units are days, and at this temperature development is completed on about the fifteenth day. If the value of the time unit is coördinated with the temperature coefficient, the cone becomes a constant expression of growth for all temperatures. The growth curve is the usual sigmoid characteristic of developing organisms in general.

At a given temperature, development to the complete functional stage has a specific duration. Equally striking is the subsequent history of the individual so formed. If the temperature is 26° C. and the developmental period fifteen days, the individual lives, feeds, and grows a

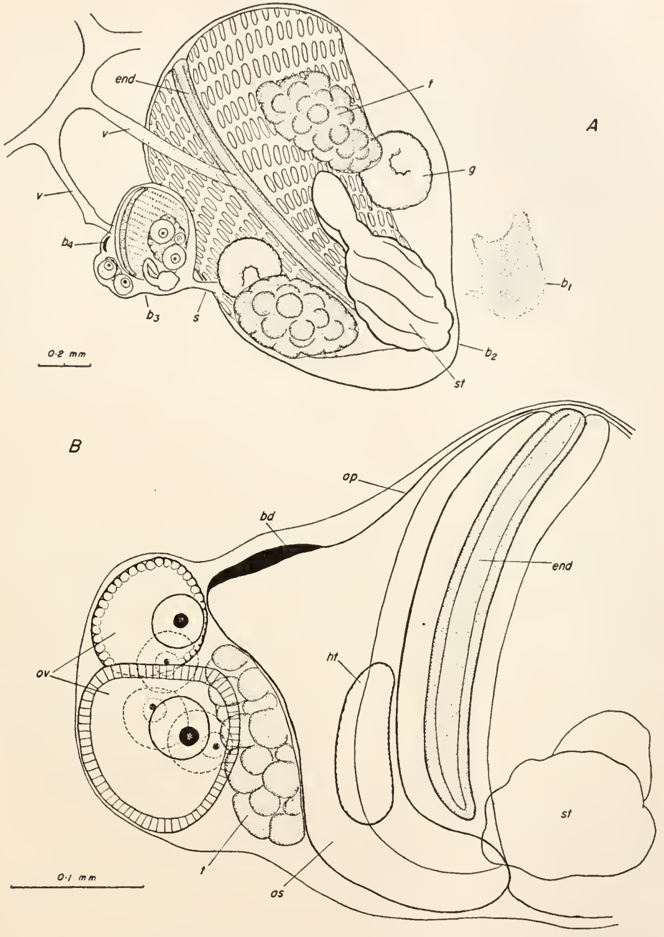

Fig. 1, *A*. Four generations representing complete cycle of zoöid. b_1, "ghost" of autolysed zoöid of preceding bud generation; b_2, ventral view of active zoöid bearing right bud only and containing developing eggs in gastrula stage. b_3, bud borne by active zoöid, with rows of definitive stigmata about to become perforate, and in turn bearing bud of next generation in its initial disc stage (b_4). Both the active zoöid (b_2) and its bud (b_3) are connected with the colonial circulatory system by their ventral ampullary vessel, v.

B. Part of b_3 at a higher magnification, showing general relationship of bud disc, atrial epithelium, and gonads. This stage is drawn at the same magnification as those of Fig. 4 and follows as a stage of bud development Fig. 4, *F*.

as, atrial sac; *ap*, atrial epithelium; *bd*, bud disc; *end*, endostyle; *g*, gastrula; *ht*, heart; *ov*, ovum; *s*, bud stalk; *st*, stomach; *t*, testis; *v*, ampullary connecting vessel.

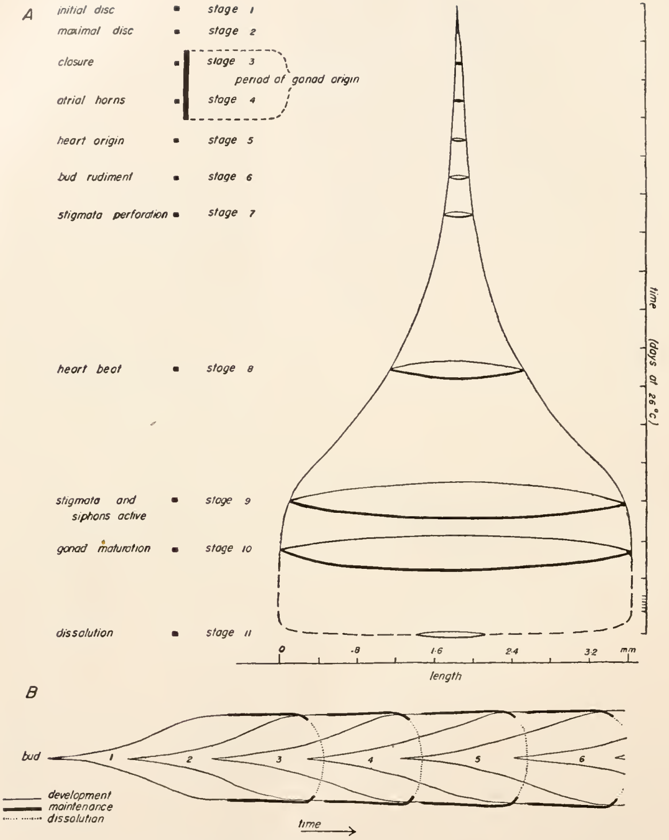

FIG. 2, *A*. Critical stages of bud development in relation to growth curve and whole life cycle of zoöid. The time scale is abbreviated for the maintenance or active period.

B. Similar growth curves for five successive generations indicating overlapping of generations and the three phases of development, maintenance and dissolution.

little, for about seven days. After this a period of dissolution and autolysis occupies a further two days. In Fig. 2, *A,* these last two phases are indicated against a condensed time scale. Their duration is related to temperature in the same way as is the first or developmental phase.

As shown in Fig. 1, *A,* the first or disc stage in bud development appears when the parent zoöid is itself but one-fifth grown and in turn still attached to its parent. In other words, there is a great extent of overlap in the life cycles of successive generations. This is shown in Fig. 2, *B*. Five complete cycles are shown in the form of overlapping growth cones related to one time scale. It can be seen that in any temporal cross-section three generations in their respective existence phases will occur. It may also be seen that the final phase of dissolution and autolysis commences immediately after the bud of the succeeding generation reaches its full size and ends at about the time the bud attains its maturity. For each generation the developmental phase is shown in light line, the mature phase in heavy line, and the dissolution phase in dotted line.

Early Development of the Bud

Throughout development the epidermis, arising in continuity with the epidermis of the parent, plays almost no part other than to form more epidermis conforming in area and shape to that of the organism arising from the atrial tissue. For the most part therefore it will be ignored.

The youngest stage so far detected consisted of eight atrial cells forming a disc of three cells, more flat than cubical, in cross-section. The whole disc was about thirty micra in diameter, and is shown in surface view in Fig. 3, *B,* and in optical section in continuity with the squamous atrial epithelium in Fig. 3, *C*. Figure 3, *A* represents an arc of the egg of *Botryllus* drawn to the same scale to show the relative size of the egg and the bud rudiment.

As the number of cells constituting the disc increases, the area of the disc increases and the constituent cells change from a sub-cubical to a columnar shape. This leads one to suspect that the columnar condition is typical but in the earliest stage the transition to the surrounding squamous atrial epithelium is so short that the columnar condition can be only partially expressed.

In Fig. 3, *D* the disc stage is shown at its maximum size. With further increase in area, or cell number, it curves into an arc, into a hemisphere, and eventually into a closed sphere (Fig. 3, *E, F, G, H*). The sphere becomes pinched off from the atrial epithelium from which it originated, and the bud remains connected with the parent primarily by an epidermal stalk. Two phases may accordingly be distinguished, an expanding disc phase, and the phase of continuing expansion during which the disc curves into a hollow sphere. A further feature of considerable significance is associated with the second of these phases. This is shown in Fig. 3, *G* and *H,* in which the gonads are already appearing. In the closed sphere stage shown in Fig. 3, *H,* the sphere proper is shown in optical section. In addition certain cells stand out clearly in surface view. These consist of three primary ova and a number of small more ventrally placed cells destined to form the testis. In the younger stage in Fig. 3, *G,* even before the sphere has closed, four cells can be seen which, from their position and shape, are undoubtedly four primary ova. These and later reproductive cells arise by extrusion or delamination from the wall of the sphere. The gonads arise therefore in a remarkably precocious manner.

In Fig. 3, *H,* the epidermis shows definite evidence of active morphogenesis, for the distal evagination is the rudiment of the epidermal stolon that unites eventually with the colonial circulatory system. The disc, hemisphere, and closed sphere stages are shown on one-half the scale of Fig. 3 in Fig. 4. In this are also shown three subsequent stages. Apart from the growth and elaboration of the gonads, which is described in detail later in this paper, these six stages represent stages in a single continuous process. The process of expansion and folding that changes a disc into a hollow sphere continues so that the sphere becomes concave along several facets or arcs. As the anterior arc continues to expand, two vertical folds appear. At the same time an evagination appears in the posterior arc. These are shown in Fig. 4, *E*. The anterior folds gradually extend posteriorly until they divide the single vesicle into a median and two lateral chambers. These three units are the central pharyngeal chamber and the pair of lateral atrial chambers (Fig. 4, *F*). At the same time the posterior evagination grows out to form the rudimentary stomach and intestine. When the primary subdivision into three chambers is complete, two small evaginations develop from the central chamber. Median and anteriorly a small bulge becomes the neural mass, while a somewhat larger evagination from the left posterior wall represents the developing heart. Therefore, apart from the segregation of lateral masses from the wall of the sphere to form the gonads, all the principal divisions of the *Botryllus* zoöid are produced, pharyngeal and atrial chambers, intestine, heart and neural complex by a simple process of progressive folding of an expanding sheet of tissue. These are all clearly shown in Fig. 4, *F*.

Later development is primarily an elaboration of detail of each of these divisions. Figure 1, *B* is of the same scale as Fig. 4 and demonstrates both the extent of growth and elaboration that occur by the time the developing atrial epithelium in turn has formed its bud disc. The

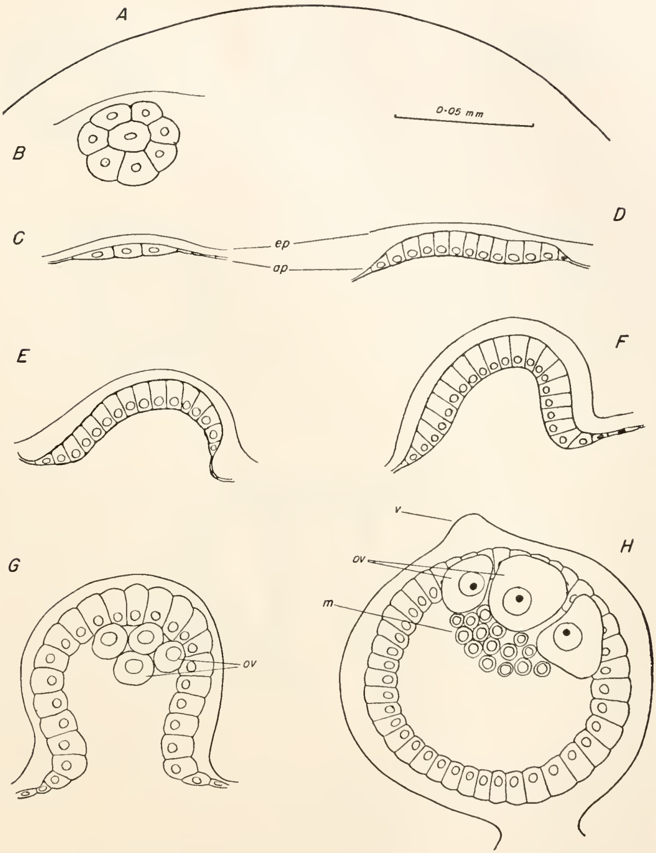

FIG. 3. Formation of bud.

A. Part of circumference of mature egg drawn to same scale for comparison of size.

B, C. Surface view and optical section of initial disc (stage 1).

D. Optical section of maximal disc (stage 2).

E, F. Arching of disc to form sphere or vesicle.

G. Bud vesicle beginning to close proximally, and showing lateral segregation of four cells destined to become mature ova, extruded into space between inner and epidermal vesicles.

H. Later stage (stage 3) with vesicle closed, epidermal ampullary vessel protruding distally, three presumptive mature ova and a number of male cells all extruded from the lateral wall of the vesicle, to lie outside it. A similar condition exists on the opposite side of the vesicle, not shown in the figure.

ep, epidermis; *ap,* atrial epithelium.

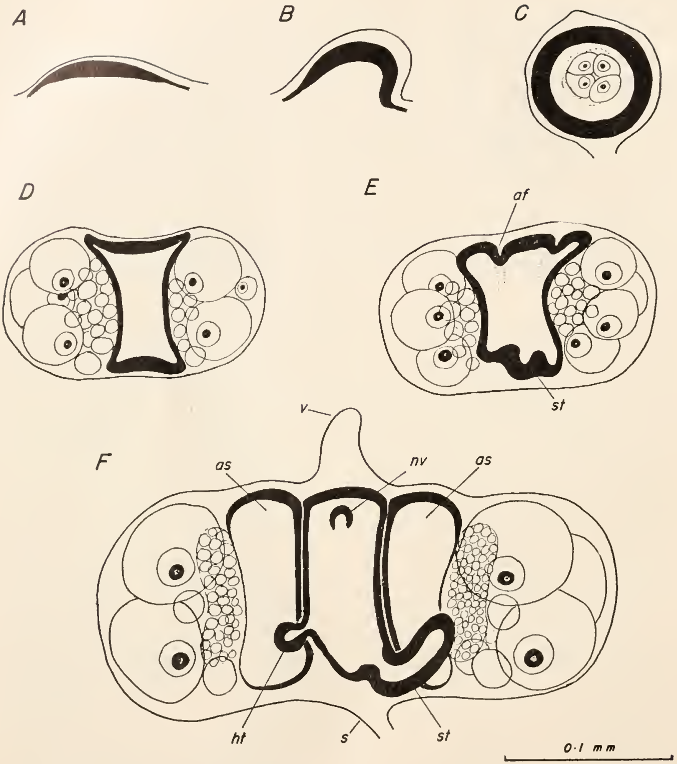

FIG. 4. Early development of bud.

A. Maximal disc (stage 2), from left side.

B. Hemisphere stage, from right side.

C. Closed vesicle (stage 3), from right side, with gonads extruded from wall.

D. Continued gonad segregation, stage viewed from ventral aspect.

E. Formation of atrial folds and intestinal outgrowth (stage 4), from ventral side.

F. (Stage 5.) Origin of heart, intestine and neural vesicle, and completion of subdivision of vesicle into central pharyngeal chamber and lateral atrial sacs, from ventral side. The succeeding stage (stage 6) is shown on the same scale as Fig. 1, *B.*

af, atrial fold; *as,* atrial sac; *ht,* heart; *nv,* neural vesicle; *s,* stalk; *st,* stomach; *v,* epidermal ampullary vessel.

bud disc appears at a precisely definable stage in the whole development and is to be regarded as an essential and definite constituent part of the organization of a specific stage.

Later Development

Development of Stigmata.—Gill slits develop as perforations of the combined pharyngo-atrial wall. This is the case both for the organism developing directly from the egg and for the developing bud. In the first case atrial sacs grow in on each side of the embryo and come into contact with the pharynx wall. Gill slits appear only within the area of contact. In the bud the equivalent double wall is formed, as already described, by the downgrowth of the pair of anterior folds that divide the primary vesicle into the central and lateral chambers, as shown in Fig. 4, *E* and *F*. The two walls are shown in Fig. 5, *A* at a stage intermediate between the preceding two. Only when this double wall expands to about ten times its linear size does stigmata formation become evident. The first indication is the appearance of an alternating thickening and thinning (spatially) of each of the two component epithelia separately, as shown in Fig. 5, *B*. The thick ridges run dorso-ventrally from the mid-dorsal line to the endostyle and each represents a row of stigmata. Between the ridges the epithelia flatten out as the interstigmal tissue. This condition is definitely associated with the stage bearing the bud disc stage of the next generation.

As the two layers of ridges or thickenings increase somewhat in depth they come into contact and fuse at a series of points along each pair of ridges. Perforation occurs at these points to form the rows of stigmata in their first definitive stage. The first perforate stage is shown in Fig. 5, *C* and *D*. Subsequent development consists of an elaboration of each of the units thus formed. No more will be added. Perforation of the fusing wall occurs at a definite and precisely definable stage of development. At this same stage other features of the developing pattern will be at a constant associated condition.

The stage of development of the bud of the next generation conforms to this relationship just as any other feature, and is near the hemisphere stage of vesicle formation (Fig. 3, *F* and *G*). In other words, the bud itself is an integral part of the whole organization pattern and the time and place of its inception are as sharply defined as that of any other unit structure in the developing organism.

At perforation each stigma in surface view consists of a rosette of about six cells (Fig. 5, *D*). Each constituent rosette of a row continues its development as a unit. With multiplication of its seemingly unspecialized cells the central aperture bordered by the cells expands and elongates, so that the size and length of each stigma increase progressively. When the cell-multiplication is almost terminated each cell develops short cilia. Further growth of each stigma to approximately

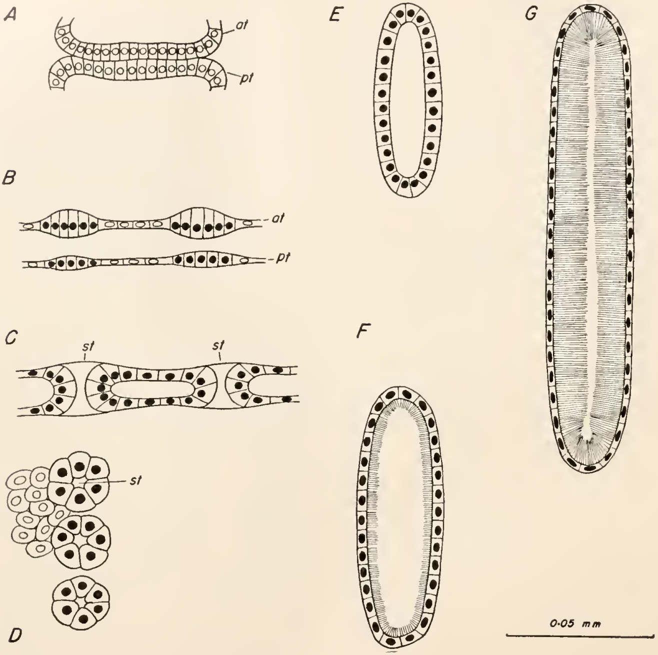

Fig. 5. Development of gill slits (stigmata).

A. Double layer formed of inner atrial wall and of pharyngeal wall.

B. Alternate thickening of atrial wall and corresponding thickenings of pharyngeal wall, each such paired thickening representing cross-section through ridge destined to become row of stigmata.

C. Equivalent section to *B,* but of perforate stage.

D. Surface view of *C,* showing three stigmata rosettes in initial perforate condition.

E, F, and *G.* Three stages in subsequent growth and differentiation of a single rosette to form a functional gill slit.

at, atrial wall; *pt,* pharyngeal wall; *st,* perforate stigmata.

double its size takes place as the result of change in cell size and shape, and at the same time the cilia grow until they almost touch those from the opposite side (Fig. 5, *F* and *G*).

Accordingly the following features are evident in the development of the gill slits: There is a primary condition in which atrial and pharyngeal epithelia are present and in virtual contact. The basic pattern is expressed as a series of ridges, each with a series of swellings in each tissue some time before stigmata formation. Perforate stigmata appear at the points of fusion between the two tissues, while subsequent growth consists first of a period of cell multiplication and then of a terminal phase of individual cell expansion and cyto-differentiation.

Development of the Gonads.—The origin of the gonads has been described already. Their subsequent development as a unit organ is, however, of some interest, as is that of a single ovum. Figure 6 shows sections through a number of stages. Figure 6, *A* represents a section of a stage immediately following that seen as a whole mount in Fig. 3, *H*. The originally thick wall of the internal vesicle is divided into the thin atrial wall and the massive developing gonad. The gonad here consists of two primary ova and a mass of loose cells representing a few rudimentary ova and many male cells. Cells are added to the collection over a considerable period from some parts of the inner retaining wall. In other words, as the lateral walls continue to grow, the splitting into inner atrial and outer gonadial components continues in marginal regions previously incapable of such splitting by virtue of insufficient cells. This is shown in Fig. 6, *B*, a section passing transversely across the anterior end of a bud at a considerably later stage, a stage intermediate between those shown in Fig. 4, *E* and *F*.

The section shown in Fig. 6, *C* illustrates several points of interest. The inner atrial epithelium is entirely distinct in kind and in space from any part of the gonad. The form of the lobular testis becomes apparent in spite of the small number of its constituent cells. And in the case of both ovary and testis there is a residual mass of cells unincorporated into those organs. In the case of the ovary, the small inner ova never grow and mature. The residual cells of the testis may or may not develop into testicular lobes, depending on the degree of belatedness of their segregation. Virtually the complete form of the testis is to be seen in the stage represented in Fig. 6, *E*, even though the testis here is less than one-quarter its final size (in linear dimension). The form is almost fully expressed, but its histo-differentiation is indiscernible. In fact the final differentiation into condensed and tailled spermatozoa occurs only after the full size of the developing bud is at last attained.

In the ovary those ova segregated from the vesicle wall in the first phase of gonad formation (in number from one to four) grow and mature. Those formed later remain close to the size at which they were segregated. The primary ova, as far as can be determined, in-

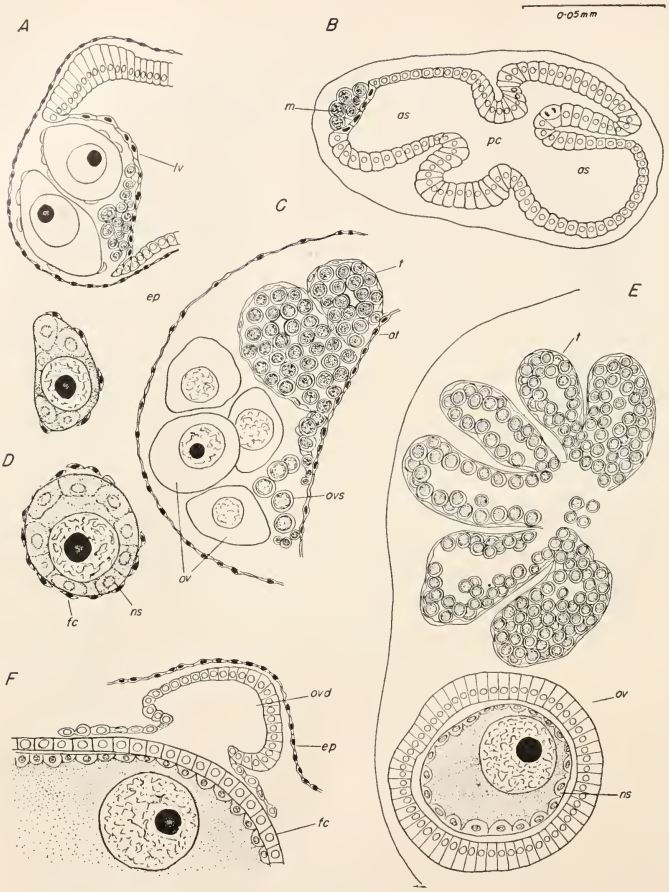

FIG. 6. Development of gonad. All drawings from actual, not merely optical, sections.

A. Left half of advanced vesicle (between stages 2 and 3) showing differentiation of lateral wall into atrial epithelium, male cells, and presumptive mature ova.

B. Frontal section of later stage (between stages 4 and 5) in anterior region, showing continued segregation on left side of male cells from the lateral wall.

C. Left side of bud at stage 5 showing four presumptive mature ova, a few undeveloping ova, and precocious lobulation of testis.

D. Two isolated ova with follicle and nurse cells.

E. Gonad of stage 6, showing lobular testis, and a single ovum with numerous follicle and nurse cells.

F. Part of ovum at its maximum size, showing nurse cells, follicle cells, and developing oviduct of same origin as follicle cells.

at, atrial epithelium; *ep,* epidermis; *fc,* follicle cell; *lv,* left wall of primary vesicle; *m,* male cells; *ns,* nurse cells; *ov,* ovum; *ovd,* oviduct; *ovs,* secondary ova; *t,* testis.

clude other cells from the first. No stage, with the possible exception of those shown in Fig. 3, has been seen in which the ova proper are without accessory cells. These cells are of two kinds, a few flattened follicle cells clinging to the surface, and an equally small number of nurse cells completely within the cytoplasm of the ovum. Following multiplication, the outer surface cells become columnar, as in Fig. 6, *D*, and eventually flatten again as they give rise to the egg chorion. The nurse cells also multiply and are clearly involved in the growth of the ovum. They are eventually extruded into the perivitelline space as the inner follicle cells or "test" cells of the mature ovum (cp. Berrill, 1929).

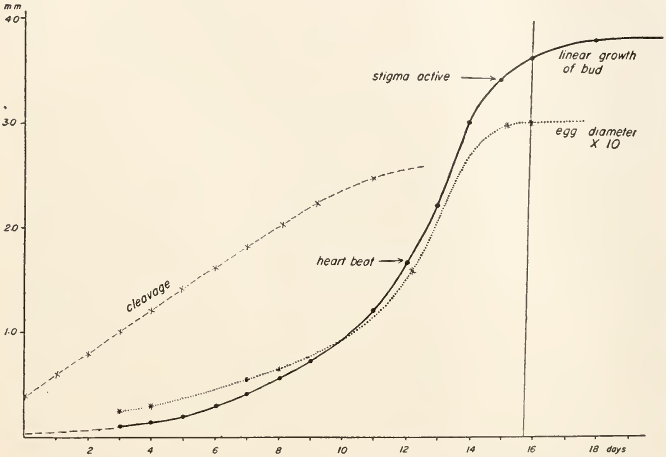

FIG. 7. Growth curves for linear growth of bud, and of single egg × 10. On the same chart is also shown a cleavage curve indicating the geometrical increase in cell number associated with growth.

Thus the development of the gonad as a whole is a comparatively complex process. Yet the ova and spermatozoa attain actual functional maturity at virtually the same time and almost at once after the bud as a whole has become active. Either some factors external to the gonads suddenly terminate growth and multiplication and enforce final differentiation to coincide with that of the rest of the bud, which is unlikely, or the development has from the first been approaching a condition of equilibrium permitting final cyto-differentiation. That this last is the case is shown by a comparison of the growth curves for the bud as a whole and of a single ovum. The curves for the linear growth of the bud and the ovum, when reduced to equivalent scales, are practically identical, as may be seen in Fig. 7. Both are sigmoid curves indicative of an approach to and attainment of a " steady state."

Cell Division and Differentiation.—In all tissues of which the cells finally exhibit a marked degree of structural specialization or differentiation, the structural details become visible only at or toward the close of the phase of cell multiplication. The time at which this occurs varies greatly. In the case of spermatozoa, condensation and elongation occur only after the rest of the organism has as a whole become functionally active. In the case of ova, as distinct from their associated follicle cells, growth and differentiation without division occupy almost the whole developmental period. Muscle cells of the heart and body wall stop dividing and become greatly elongate when linear growth of the whole organism is little more than half complete. Cessation of division and subsequent formation of long cilia in stigma cells occurs very late, but is complete before elongation of spermatozoa commences. In the case of ova and muscle cells, at least, there is very considerable growth after cell division has come to an end. Growth of the organism as a whole accordingly conforms to a typical curve in spite of the fact that the growth is in part due to cell multiplication and in part to cell growth without division. The growth curve for a single ovum is similar to that of the whole organism. It seems clear that the developing bud grows at a rate characteristic of an approach to a " steady state," and that the growth of the parts, whether based on cell division or not, is not a group of independent processes coöperating to form the whole, but must be governed by the whole. Cell division as such becomes, in this view, a condition and tool of the whole developmental process rather than in any sense a basic cause. Otherwise the coördination of the varying times at which different cell types cease division and differentiate becomes virtually unaccountable. In a comparison of the growth of a non-dividing ovum with the growth of a group of cells by multiplication, it appears that the rate of volume-increase is quite independent of division processes.

Summary and Conclusions

The bud arises as a disc-like thickening of the anterior atrial wall, consisting of a small number of columnar cells transformed from the atrial epithelium, overlain by an equivalent area of unmodified epidermis. The polarity of the disc and subsequent organism is an extension of that of the parental tissue, with regard both to the antero-posterior and lateral axes. Development itself is fundamentally extremely simple and direct. After the completion of development there is a phase of functional activity and a phase of autolysis and dissolution. For any given time-temperature scale the duration of these last two phases is as specific and determined as that of the developmental phase. Of the two tissues constituting the bud disc the epidermis forms only more epidermis, though acquiring the form of a whole organism including the ventral stolonic outgrowth. The atrial component of the disc forms everything else. As the disc expands, by means of cell multiplication, it transforms progressively into a hemisphere and eventually into a hollow sphere attached by a narrow stalk to the parental tissue. Two folds develop anteriorly and divide the vesicle into two lateral and one median chamber. The lateral divisions represent the atrial chambers, the median the pharyngeal sac and from it three evaginations are formed representing the heart, neural mass, and intestine respectively. Later development is primarily an elaboration of these unit-regions. As an example, the formation, growth and differentiation of gill slits in the pharyngeal wall is described in detail. The essential pattern of the stigmata is apparent even before they become perforate. Each stage in their development is precisely correlated with specific stages in the development of the whole organism.

The bud anlagen of the succeeding generation appear as discs in the anterior wall of the left and right atrial chambers at a specific stage in the development. This stage is that in which rows of stigmata, while not yet perforate, are represented by ridges or folds of the pharyngeal wall. At the time of perforation, the buds are approximately at the closed vesicle stage. The buds, in fact, are to be regarded as essential constituents of the organization pattern, appearing and developing in time and place in a manner strictly analogous to that of any other unit structure.

The gonads segregate as a mass from the lateral walls of the bud at an extremely precocious period, even while the primary vesicle is in process of formation. Once segregated, they in turn develop as a seemingly independent unit structure. The testes show the final lobular form virtually as soon as sufficient cells are present for its expression. Ova, apart from the associated internal and external follicle cells, grow and differentiate without dividing. They mature finally at the same time as the spermatozoa which cease dividing and differentiate later than any other tissue of the bud.

The development of each tissue is fundamentally the same. A period of cell multiplication is followed by a phase of final differentiation. This last phase may or may not include a period of cell enlargement, depending on the cell type to be formed. In the case of ova the multiplication phase is barely present at all and the second phase occupies most of the developmental period, involving enormous growth. In spermatozoa the case is reversed and the final phase is extremely brief and actually involves reduction in cell size. Muscle tissue lies between these two extremes, while most other tissues approach more the condition of spermatozoa.

The whole development of the bud and that of its component parts is therefore as direct a process as can be conceived, without there being any indication of the divergence to form tadpole larvae associated with egg development. Cell multiplication continues to a greatly varying extent in different parts and tissues, while the linear growth of the whole or of a non-dividing ovum follows a regular sigmoid curve typical of an approach to and attainment of a "steady state." In fact, the development of the bud is essentially such a unitary process that "wholeness" can be said to be the most outstanding feature of the organism not only in its final functional phases but of every moment of its existence, and especially of the beginning. It is virtually as though organization is present from the first, though the extent of its visible expression is closely correlated with and limited by quantity of available material at every moment of development.

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SIZE AND MORPHOGENESIS IN THE BUD OF BOTRYLLUS

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The bud of *Botryllus* first appears as a disc arising in the anterior wall of each of the atrial chambers. The disc grows a little and then transforms into a sphere. The size to which the disc grows before it starts to transform varies among different bud generations in a *Botryllus* colony, increasing slightly with each successive generation of buds. The present account is primarily a comparison of the development of the buds arising from the smaller discs of early generations with the large discs of late generations, within the colony formed from a single fertilized egg. In addition to differences in size of bud primordia from early and late generations in the colony, there are usually size differences between the bud primordia of the right and left sides, that of the right side being the larger.

The development of a bud has as its basis a continuous material expansion from the small group of cells constituting the primary disc to a functional bud of several thousand times its volume and cell number. The significance of this expansion is paramount. As the disc expands in area it becomes curved into a hollow sphere. As the sphere expands, its surface folds inwards to divide it into three chambers, the major territories of the body. With continued expansion, further surface folding occurs to divide off smaller territories such as neural mass, heart, and intestine. It can be said that for each successive size the material (mass, area, cell number, etc., however it may be expressed) present at that moment expresses virtually every character of the final organization that is not inhibited by the limitation of size itself.

Each bud disc arises from the atrial epithelium as a group of cells that gradually acquire a columnar form. The epidermis forms an equivalent overlying component of the disc, but plays a relatively minor part in the subsequent development. Since every disc of atrial cells has to develop from the general atrial epithelium, there is almost certainly no real minimum size that can be compared in different generations. On the other hand, the disc in every case grows to a certain extent before changes in form begin, and the size or cell number of the disc at its maximum size, which is a precise stage, is a value readily compared.

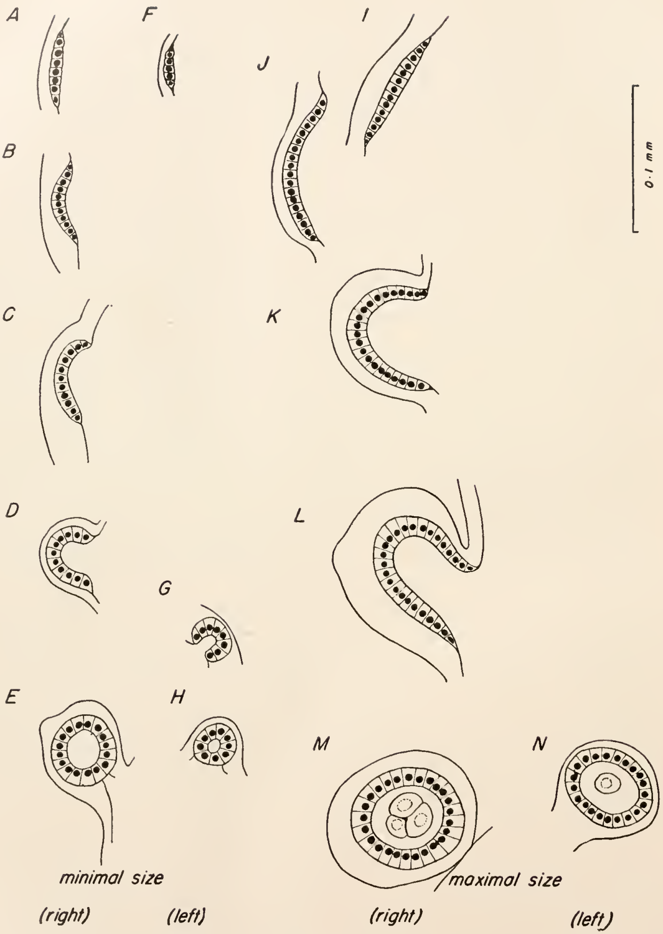
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FIG. 1. Formation of bud vesicles, all in optical section. *A, F,* and *I* are three maximal bud discs. *A–E* represents vesicle formation from small right maximal disc from zoöid of young colony. *F–H,* the smaller left maximal disc of same series. *I–M,* vesicle formation from large right maximal disc from zoöid of a mature colony. *M* and *N,* right and left vesicles from same individual and forming three and one mature ova respectively.

The smallest maximal disc commonly seen consists of about six cells in optical section, the largest of about fourteen cells, or a difference of about eight times in volume of tissue or number of cells present at this stage. Figures 1, 2, and 3 represent a comparison of the development of two sizes of maximal right bud discs, from early and late generations respectively. In Fig. 1, *G, H,* and *N,* the smaller left buds are also shown. In this figure several features of comparative interest are clear. The relative difference in size of three maximal disc stages is maintained in the subsequent stages of hemisphere and sphere. In optical section these maximal discs have 5, 8, and 14 cells respectively (Fig. 1, *F, A* and *I*), representing totals of about 21, 48, and 150 cells (ratios 1:2·1:7·1). In the corresponding closed sphere or vesicle stages, optical sections show 9, 15, and 25 cells respectively (Fig. 1, *H, E* and *M*), representing cell totals of about 33, 75, and 210 (ratios 1:2·2:6·3). From these values two facts emerge. The ratio of cell numbers representing the smallest and largest maximal disc illustrated is about 1:7. The same ratio holds for the closed vesicle stage, and it is evident that whatever the size of the maximal discs, the transformation is correlated with an increase in cell number of about one and one-half times that of the disc.

Morphogenesis is thus independent of absolute cell number, but closely dependent on relative cell number.

In Fig. 1 two other features are evident. The relation of morphogenesis to cell number is the same in the epidermis as in the atrial tissue. The epidermis conforms in size and shape to the inner tissue, and as may be seen in Fig. 1, *E* and *K,* the protrusion foreshadowing the ventral ampullary vessel appears in both small and large vesicle stages, and in spite of the very early stage in development as a whole.

It is of greater interest that gonad tissue is segregated from the right and left lateral wall of the vesicle stage in the large forms but not in the small. These two correlated variations in the vesicle stage, namely degree of segregation of gonad tissue and the absolute size, produce an increasingly marked effect on later stages of development. Figure 2 shows immediately succeeding stages drawn to the same scale as those in Fig. 1. Figure 3 shows still later stages at necessarily reduced scales. In each case equivalent stages are shown for the development of both large and small primordia.

The two primary differences, in size and gonad development, are maintained in an increasingly obvious form. Thus in Fig. 2, the three stages *A, B,* and *C* are morphologically equivalent to the stages *D, E,* and *F.* In *A* and *D* the folding of the vesicle wall to delimit the primary divisions of the body are just beginning. In *B* and *E* they are com-

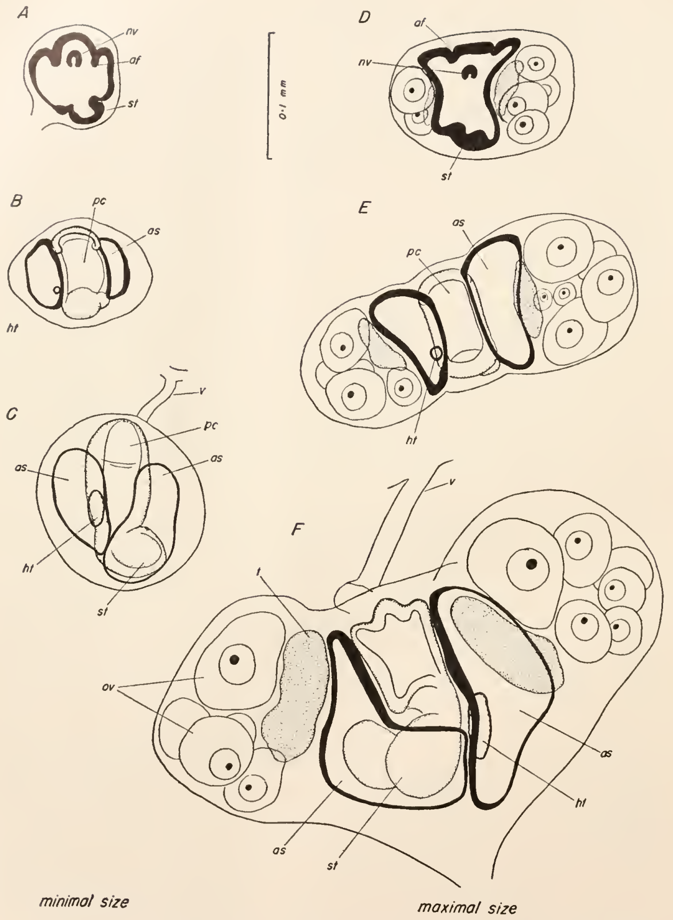
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FIG. 2. Development, at same magnification, of series *A–E* and *I–M* of Fig. 1. *A–C,* development of small vesicle, *D–F* of large vesicle, showing differences in size of equivalent stages and in presence and absence of gonads.

af, atrial folds; *as,* atrial sac; *ht,* heart; *nv,* neural vesicle; *ov,* ova; *pc,* pharyngeal cavity; *st,* stomach; *t,* testis; *v,* ventral ampullary vessel.

pleted, and atrial chambers, pharynx, intestine, heart, and neural mass already exist as unit regions. The difference in diameter of the closed vesicle stage shown in Fig. 1, *E* and *M,* is fully maintained. In addition there is the striking difference in gonad development. In the series of stages associated with the small primordium none appear, in the larger they continue to develop and become massive organs on each side between the lateral wall of the atrial epithelium and the epidermis. These differences become more and more pronounced, as may be seen in Fig. 3.

Size and General Organization

While the structural consequences of the primary difference in size become progressively more obvious, one feature needs to be emphasized strongly. The great difference in cell number constituting maximal disc stages is maintained at least in the later closed vesicle stage, the increase being about one and one-half times. The difference in cell number is expressed less markedly in disc and vesicle diameters. In the two main series illustrated in Fig. 1, the diameter of the larger series is about one and two-thirds that of equivalent stages of the smaller series. Excluding gonads for the time being, this difference in linear dimension of the two series is maintained closely in the later stages shown in Figs. 2 and 3 up to and including the active functional stage. Not only is the linear size difference maintained throughout development, but it is equally expressed in the number of such multiple structures as stigmata. In the stages in which stigmata are just becoming perforate and in which they are active organs, the number of rows of stigmata is six in the smaller buds, ten or eleven in the larger, while the number of stigmata per row in the smaller is 12 and in the larger 22. The number of stigmata does not change during development. Thus in the two series the number of stigmata formed is proportionate to size, since both linear dimension of the whole, and the number of rows of stigmata and number of stigmata per row, vary as one to about one and two-thirds.

The difference in whole size of equivalent stages, which is expressed numerically in multiple organs such as stigmata, applies equally to organ size. This is the case for the heart, for in the three stages—primary heart vesicle, initial beating, and final—the same relative size differences are maintained.

Since the relative difference in size between the two series is maintained virtually at a constant level for all stages, it follows that each stage represents a certain degree of expansion in terms of a preceding stage, whatever may be the absolute size. A specific degree of expression of the complete organization is correlated with a certain size or

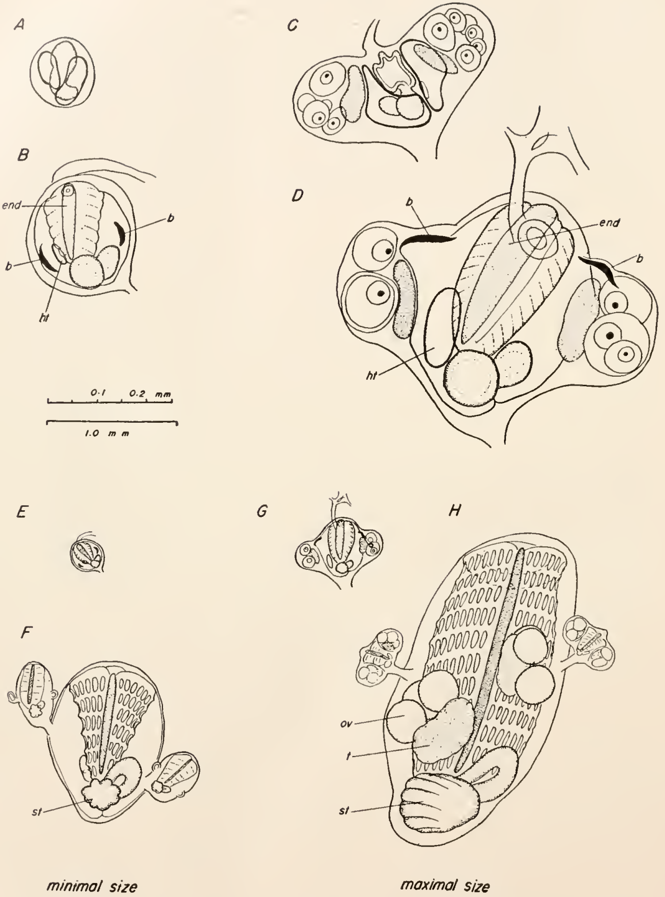
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FIG. 3. Continuation of same two developmental series, at two smaller magnifications. *A–B, E–F* continued development of smaller bud disc and vesicle shown in Figs. 1 and 2. *C–D,* and *G–H,* continued development of larger vesicle.

For purposes of comparison, *A* and *C* represent at the lower magnification the stages shown in Fig. 2, *C* and *G;* in the same way *E* and *H* are reduced from *B* and *D*. *B* and *D* are equivalent stages and have formed maximal bud discs. *E* and *H* are also equivalent and have become active zoöids. The difference with regard to size, presence of gonads, and length and number of rows of gill slits is obvious.

b, bud disc; *end,* endostyle; *ht,* heart; *ov,* ova; *st,* stomach; *t,* testis.

material quantity. This size is not absolute and is not expressable in actual measurement or cell number, but must be expressed in terms of reference to the absolute size of the maximal disc stage. This is highly significant and will be referred to again.

Development of Gonads and Initial Size

Confining the present account to the two extremes already illustrated, there is a spectacular difference in the condition of the gonads in the two series, one producing mature gonads and the other none. This difference goes back to the first stages of development. In the larger series, the gonads are separated or extruded from the lateral walls of the primary vesicle even before closure is complete. Once separated, the gonads develop apparently as independent unit regions. The separation phase is comparatively brief, and there is no tendency to form gonads except during this precise phase of the whole development. The ova destined to become mature are the first tissue to be separated, testis and prospective immature ova separating a little later.

In the smaller series, no separation of gonads occurs at all during the equivalent phase, and no gonads appear at any later time. Consequently the massive lateral bulges representing the growing gonads in the developing buds of mature colonies are absent altogether in those of very young colonies.

The correlation of presence or absence of gonad separation (and therefore of subsequent development) with the size of the transforming disc stages suggests at once that size itself may be the determiner.

Gonad tissue is separated during a very definite and specific period of bud development, namely, during a period starting before closure of the primary vesicle and lasting until the vesicle is more or less subdivided into its three primary regions. At no other moment in development, either earlier or later, is there any indication of gonad formation. There is no absolute proof that at no other time under any conditions can gonad tissue be separated, but it is reasonably certain that the capacity to produce gonad tissue is definitely limited to the period or phase in which production always occurs. In other words, the gonad primordium is determined and formed at as precise a period in the whole development as is the case for other organs, such as the heart.

Accordingly, if this assumption is made, it is easy to account for the suppression of gonad formation in the development of small buds. The situation is clearest if one compares the largest and smallest of the four vesicle stages shown in Fig. 1. In the largest, three prospective mature ova have separated from the vesicle wall. The extrusion of these particular cells commences immediately after the attainment of the open hemisphere stage. The gonad material thus separated must represent a certain minimum proportion of the lateral wall from which it arises, of the order of one-quarter to one-half. It is separated when the vesicle cells total about 160 and when there are about 22 to 26 cells in optical section (Fig. 1, *M*). At this stage, in other words, the material from which ova are separated is in the form of a sufficient number of cells for individual cells representing individual ova to be pushed out.

In the equivalent stage of the smallest series, only 7 or 8 cells constitute the optical section, and the region from which gonads and atrial wall should be differentiated consists, on each side, of only 2 cells in optical section. Accordingly the region constituting the prospective ova is at this stage and size inadequately cellular and the separation of ova at this moment becomes mechanically impossible.

Gonad formation, however, is not momentary but occupies a period of time. In the smallest series even the late closed vesicle stage consists of so few cells (Fig. 1, *E*) that at no time during the proper period can gonad tissue be separated. In the largest series the prospective mature ova are extruded before closure, male tissue and prospective immature ova during and shortly after closure of the vesicle, and some additional male cells after completion of the extrusion of female cells.

In series of developing buds of intermediate size all conceivable types of immature gonads should be found. This is the case. In a series slightly larger than the smallest, a sufficient size or cell number is attained before the gonadial phase is completely passed, and some male tissue is separated at the end of that phase. In a somewhat larger series again, and a little sooner, more gonad tissue is separated consisting of prospective immature ova and male tissue sufficient to develop into a lobular testis of submaximal size. Similarly, in a larger but not maximal series, there may be a separation at or before closure of one or two or three prospective mature ova. In other words, a complete grading from none to mature 4-ova gonads exists, correlated both with size of series and with time of separation.

Conclusions and Summary

In successive bud generations within a colony there is a progressive increase in the size of the bud rudiments and zoöids subsequently developing from them. Dealing with material derived from a single fertilized egg, it is possible to determine the relationship or importance of rudiment size to morphogenesis. Development in every case is simple and direct. It consists of the growth of the rudiment to a maximal disc stage, the conversion of the disc into a sphere, the subdivision of the sphere or vesicle into unit regions, the whole process being accompanied and conditioned by expansion or growth of tissue.

The maximal disc is a precise relative stage. The larger discs, typical of late and mature generations, may be seven times as large in area as those of early generations.

Whatever the size of the maximal disc, succeeding stages bear to it definite growth ratios. A certain percentage expansion or growth of the disc tissue is correlated with a specific developmental stage, whatever the absolute sizes may be. Absolute size must be determined during the initial phase of development before the disc exhibits any tendency to transform into a vesicle.

Ultimate size being thus initially determined, there is variability in the following expressions of size. All organs and regions vary in absolute size, maintaining their proportionate dimensions relative to the whole. Multiple structures, such as stigmata, vary little in absolute size for a given stage but vary in number in proportion to tissue area. The relatively massive gonads, fully formed only in the largest series, are partially or completely inhibited in the smaller.

Gonad formation is essentially a serial separation of the various components during a short phase of development, lasting from the open hemisphere stage to the expanded closed vesicle stage. If the size of the whole permits separation of each component as discrete cells at the proper time for separation, maximal mature gonads will be formed and develop. If size is so reduced that the various components cannot be materially separated as cells, separation is inhibited and no gonads will develop at the normal or any other time. With successive increases in size from this last condition an adequate cellular state is reached, at first including the later phases of the gonadial period and progressively including the earlier, so that a series of immature gonads appear in the inverse order of normal maximal development. Prospective mature ova do not appear at a time normal for the appearance of prospective immature ova or for male cells. Gonad components that do not separate at their normal time do not appear at all.

THE EFFECT OF SALINITY UPON THE RATE OF EXCYSTMENT OF ARTEMIA

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INTRODUCTION

The phyllopod crustacean *Artemia* lives and reproduces in natural and artificial brine pools and lakes in many parts of the world. It tolerates an extreme range of salinity, pH, and other environmental conditions, although it is relatively intolerant of certain substances such as potassium[1] (Martin and Wilbur, 1921; Boone and Becking, 1931). *Artemia* does not require brine, or other environmental extremes, since it completes the life cycle in ordinary sea water in the laboratory, but it is defenseless in nature and is quickly destroyed by predators except in environments which exclude them.

Artemia are abundant in evaporating ponds on the margin of San Francisco Bay where salt is manufactured from sea water by solar evaporation. This particular *Artemia* has been regarded as a variety of *A. salina,* and also as a separate species, *A. franciscana.* Bond (1932) suggests, after experiments on the effect of salinity on development, that it should be regarded as a separate species.

The *Artemia* from the margin of San Francisco Bay reproduce by two methods. In the presence of males the same females sometimes produce viviparous nauplii, and at other times they release encysted embryos which are encased in hard chitinous coverings or shells. These encysted embryos will not ordinarily hatch in sea water until they have first been desiccated. Air-dry cysts remain viable for many years and when they are placed in sea water, the embryos hatch as swimming nauplii.

Dry cysts are approximately ⅕ mm. in diameter. They are deeply indented on one side, but when they are placed in sea water, or in sea water of modified salinity in which they will hatch, they take up water and round out to become spherical. After a time, which depends among other things on salinity and temperature, the chitinous cyst wall or shell splits, and the embryo emerges head first encased within a delicate transparent membrane or sac. This sac may remain attached at one end to the shell or it may at once be free. During the emergence from the shell and for some time thereafter the nauplius is quiescent within the sac. The sac finally begins to soften and dissolve and the nauplius moves its appendages. The nauplius completes its excystment by hatching or escaping from the remains of the sac, after which it swims actively about. At the time of hatching the nauplius contains an appreciable supply of yolk and even in the absence of food it develops for several days and undergoes the first moult to form a metanauplius. The external anatomy of the developmental stages is completely described and figured by Heath (1924).

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[1] This intolerance of potassium appears to be an important factor in the distribution of *Artemia* in desert salt lakes (Boone and Becking, 1931).

The two stages or actions in the excystment, the initial emergence from the shell, and the final hatching from the membranous sac, will for convenience be referred to as " emergence " and " hatching " respectively. Both emergence and hatching proceed rapidly compared with the time lapse before emergence and between emergence and hatching. For accuracy in determining rates, it is necessary to define these two stages rather precisely even if somewhat arbitrarily. The emergence from the shell is a discreet abortive process and a nauplius is considered emerged if the eye can be seen. The first indication of hatching from the sac is usually the projection of the first pair of antennae. Soon the large second pair is also projected outside the sac and swimming or attempts at swimming begin. The first movements of the appendages are often intermittent and uncoördinated. A nauplius is considered hatched when the first two pairs of appendages project outside the sac and are motile. The third pair of appendages soon slides out and the remnant of the sac is left behind.

The effects of specific ions and of ion antagonism on the excystment of *Artemia* have been studied by Boone and Becking (1931), who also have concluded that osmotic pressure has much less effect on excystment than chemical factors. Jacobi and Becking (1933) observed that excystment will not take place in natural sea water concentrates of three or more molar equivalent. The present experiments were undertaken to test the effect of total salinity on the rate of excystment in diluted and concentrated sea water in which the proportion, or relative concentration, of the ions contained in sea water remains essentially unaltered. In the strongest concentration used (225 per cent sea water), there was no visible precipitation of any kind of salt so that the ionic proportions were unaltered except for second order differential effects on dissociation, and minor pH effects. The minimum salinity in which emergence, hatching, and early development will take place has also been determined.

METHOD

The Cysts

The cysts used in these experiments were generously provided through the courtesy of Dr. Alvin Seale of the San Francisco Aquarium Society. They were collected near Redwood City, California, June 10, 1937, and the experiments were carried out in the winter of 1939.

About 20 per cent of the original sample of cysts excysted in sea water. It was found by dissection that most of the remainder were empty shells of previously excysted embryos, although some contained embryos which were presumably dead. The empty shells are difficult to distinguish by simple inspection, but it was found that they could be partly separated out by rapid differential flotation since they contain air until they have soaked. The cysts were shaken and suspended in distilled water in a test tube and those that floated were discarded. Some good cysts were discarded by this method, and not all that sank were good cysts, but a stock was obtained in which the percentage which excysted had been increased from 20 to about 60. In this process the cysts were exposed to distilled water for only three minutes and were then dried on filter paper for five days at room temperature and 30 per cent humidity. After this they were stored for some time in a stoppered bottle before using. The brief washing in distilled water also served to remove most of the salt on the cysts, which is important for the present purpose. Otherwise, the differential flotation can be carried out as well in sea water (Whitaker, 1940). The rate of excystment in normal sea water varies with the duration of drying and storing after the washing, and would no doubt differ in different samples of cysts for this and other reasons. The present experiments were carried out on a single stock of cysts during a period in which the rate of excystment in normal sea water was practically constant.

The Media

Sea water (specific gravity, 1.025, pH 7.9–8.0) was collected at Moss Beach, California, and was filtered before being concentrated or diluted. The specific gravity 1.025 was taken as a base throughout and was considered to represent the salinity of what is called 100 per cent sea water. Sea water was diluted by adding triple glass distilled water to prepare the dilutions shown in Table I. It was concentrated by evaporating under reduced pressure in a water bath at 45–50° C. The resulting brine was diluted back with distilled water to prepare the salinities greater than sea water which are also shown in Table I. Specific gravities were checked with pycnometers. The concentrating process removed gases from the brine so the solutions more concentrated than sea water were re-equilibrated by aerating for several hours with a sintered glass nozzle. A glass electrode was used to measure pH. The rate of excystment is practically unaffected by pH within the range 8.3–7.7, so that pH can hardly be an important factor in the present instance except perhaps in distilled water (see Table I).

Excystment

Small 1 cc. Syracuse dishes were used for excystment. Especially in the solutions of high salinity, the cysts tend to float and to accumulate in the meniscus where observation is difficult. Accumulation in the meniscus was prevented by dipping the dishes in hot, pure high melting point paraffin. A thin coating of paraffin causes the water meniscus to be inverted. In each experiment about twenty cysts were placed in 1 cc. of medium in each small dish, and ten small dishes were placed in Petri dishes arranged as moist chambers to prevent evaporation. The two most dilute solutions (0 and 12½ per cent sea water) were changed once in the course of the experiments so that the small amount of salt on the cysts would not appreciably alter the salinity. No measurable changes of salinity took place during the experiments. The moist chambers and the solutions were kept throughout in a humid constant temperature room at 25 ± ¼° C.

TABLE I

Salinity and pH of media. For convenience in comparing, salinity is expressed as a percentage of the salinity of normal sea water (specific gravity 1.025), and a solution is described in terms of its relative salinity as the corresponding percentage of sea water.

Percentage Sea Water	Specific Gravity	pH
225	1.0562	8.0*
200	1.0500	8.2*
175	1.0438	8.3*
150	1.0375	8.3
125	1.0312	8.2
100	1.0250	8.0
75	1.0187	8.0
50	1.0125	7.9
25	1.0062	7.9
12½	1.0031	7.7
0†	1.0000	6.5

* Probably inaccurate due to effect of high salt concentration on glass electrode.
† Distilled water.

After emergence began in a population, counts were made of the numbers emerged and hatched at least every two hours until at least 60–70 per cent had hatched. The numbers that ultimately emerged and hatched were also determined at about 96 hours, and the original empty shells and non-viable cysts were excluded from consideration. The number emerged and the number hatched at the time of each observation were treated as percentages of the number that ultimately emerged and hatched, respectively. The percentages obtained from successive observations were plotted against time to give signoid curves, and the times at which 50 per cent had emerged, and at which 50 per cent had hatched, were determined from these curves by interpolation.

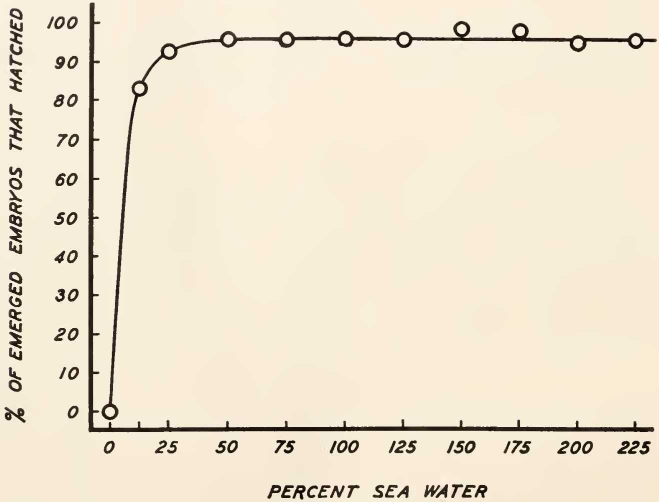

Fig. 1. The percentage of emerged embryos that hatched in diluted and concentrated sea water of various salinities. One hundred per cent sea water corresponds to specific gravity 1.025, and 0 per cent sea water is distilled water (see Table I).

Results

Five to nine experiments (involving counts on a total of 500–1,000 viable cysts) were carried out at each salinity. Throughout the range of salinities used, and in distilled water, approximately 60 per cent of the stock mixture of cysts and empty shells emerged (see method), i.e. all of the embryos which are presumed to have been viable emerged in all of the solutions. More than 96 per cent of the embryos that emerged from the shell also hatched from the membranous sac in 50–225 per cent sea water, inclusive, but in lower salinities this percentage decreased and no true hatching at all occurred in distilled water (Fig. 1). In distilled water the appendages of the emerged embryos did not move. The membranous sac disintegrated after several hours in about one-fifth of the cases, causing a sort of pseudo-hatching, but in these cases the nauplii always swelled, and often burst near the first joints of the large second antennae. Inactivity of the embryo probably interferes with hatching. Occasional cysts burst within 20–30 minutes after being placed in distilled water and aborted amorphous masses. This also occurred rarely in 12½ per cent sea water and in higher salinities as well.

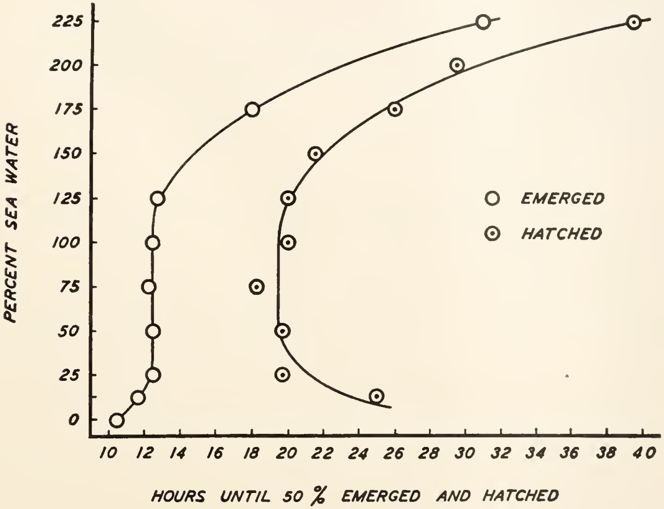

FIG. 2. The rates of emerging and hatching in diluted and concentrated sea water at 25° C. One hundred per cent sea water corresponds to specific gravity 1.025, and 0 per cent sea water is distilled water (see Table I). The curves show the time lapse until 50 per cent of the embryos in populations emerge, and until 50 per cent hatch (see text).

The embryos that hatched throughout the range 12½–225 per cent sea water were all normal, active, and viable in the salinity in which they excysted. They moulted once before dying of starvation on about the fourth day. No food was provided and no attempt was made to determine the salinity requirements of more advanced developmental stages. The effect of salinity on food organisms is a complicating factor after the yolk has been consumed.

The effect of salinity on the rate of emergence and hatching is shown in Fig. 2. Each point represents the average of the recorded time lapses until 50 per cent had emerged and until 50 per cent had hatched in the several experiments at each salinity. The results of the individual experiments were quite consistent. It may be seen in Fig. 2 that the rates of emergence and hatching are little affected by osmotic pressure within the salinity range 25–125 per cent sea water, but this is not true in higher and lower salinity.

Summary and Conclusions

1. The excystment of *Artemia* takes place in two principal stages: first, the quiescent nauplius emerges from the shell of the cyst within a membranous sac, and then later the nauplius hatches from the sac and swims actively about.

2. The excystment of *Artemia* obtained from the margin of San Francisco Bay has been studied at 25° C. in diluted and concentrated sea water over a salinity range from zero (distilled water) to 225 per cent sea water (i.e., a solution in which the salt concentration is 225 per cent of the salt concentration of sea water. No salts precipitated out).

3. The same percentage of embryos emerged from the shells in all of these salinities, including zero salinity (distilled water).

4. In distilled water the emerged embryos are motionless and they do not hatch from the sac. Some swell and burst.

5. In 12½ per cent sea water, 83 per cent of the emerged embryos hatch; in 25 per cent sea water 93 per cent hatch. In 50–225 per cent sea water 96–99 per cent hatch.

6. In 12½–225 per cent sea water the nauplii that hatch are normal, active, and viable. They moult to form metanauplii before dying of starvation (in the absence of food) on about the fourth day.

7. In the salinity range 25–125 per cent sea water, the rates of emergence and hatching are practically constant (and therefore independent of change in osmotic pressure).

8. In the salinity range 150–225 per cent sea water the rates of emerging and hatching decrease with increasing salinity, but the interval between emergence and hatching is nearly constant throughout the range 25–225 per cent sea water.

9. Emergence is accelerated in 12½ and 0 per cent sea water, but hatching is retarded in 12½ per cent sea water and is inhibited in distilled water.

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SPERM ACTIVATION BY ARBACIA EGG EXTRACTS, WITH SPECIAL REFERENCE TO ECHINOCHROME

IVOR CORNMAN

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It has long been known that the eggs of various marine invertebrates secrete substances which markedly affect the behavior of sperm. One has merely to rinse eggs in sea water and add this water to a sperm suspension to produce striking changes. These effects are classified by Lillie (1924) as activation, aggregation, and agglutination. Activation is a stimulation of the sperm, bringing them instantaneously from an inactive state (as in the testis) to a high pitch of activity. Aggregation constitutes the gradual accumulation of the sperm within a region of high concentration of various agents, and in the case of egg-secretions, appears to be a chemotaxis. Agglutination is the clumping of the sperm exposed to egg-secretions. While some properties of the substances active in egg-secretions are known (Tyler and Fox, 1940), the substances themselves have not been isolated from eggs in pure enough form that we can attribute these properties to definite chemical entities. In 1939, Hartmann, Schartau, Kuhn, and Wallenfels reported that echinochrome, the pigment which gives *Arbacia* eggs their reddish color, produces the same stimulation in *Arbacia pustulosa* sperm as does the egg-secretion itself, and is effective in dilutions as great as 1 : 2,000,000,000. An attempt was made to duplicate these results, using *Arbacia punctulata* sperm, and crystalline echinochrome kindly supplied by Dr. E. G. Ball, which he had isolated from *A. punctulata* eggs (1934). No stimulating effect could be detected. Subsequently there appeared a fuller account by Hartmann and Schartau (1939), and a report by Tyler (1939) of negative results with *Strongylocentrotus purpuratus*. Because of this, it seemed worthwhile to repeat and extend the experiments with *A. punctulata*.

Echinochrome as the Activator

In preparing the solutions, both sea water and isotonic sodium chloride were used. In sodium chloride, the sperm do not agglutinate, which sometimes facilitates comparison of sperm activity. The egg-secretions were obtained by suspending one volume of eggs in 100 volumes of sea water (or NaCl) for a half-hour. The liquid, filtered free of eggs, is generally referred to as egg-water, and is extremely effective in bringing about activation and agglutination. The concentrations of echinochrome ranged from 1 : 2,500,000,000 to 1 :25,000. At the latter concentration, the echinochrome solution is pink in color and therefore is well above the concentration of echinochrome in active egg-water, which was colorless. The activating properties of the echinochrome solutions were tested immediately after preparation, to avoid possible loss of the echinochrome through its precipitation as a calcium salt in sea water, or its decomposition in alkaline solutions.

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Sperm respond readily to differences in pH. Therefore, in testing potency of echinochrome, particular attention was given to the control of pH in all solutions used. Glycyl-glycine (.001 M) and piperazine (.001 M) were used as buffers in preference to phosphate, which tends to precipitate calcium and magnesium from the sea-water solutions (Tyler and Horowitz, 1937). In these experiments little strain is placed upon the buffer systems, and measurements with the glass electrode showed these low concentrations to be adequate. In any single series, the egg-water and echinochrome solutions, and the sperm suspensions were prepared with the same buffer and the pH was measured with a glass electrode before and after the activation tests were made, as further precaution against differences in hydrogen ion concentration. Most of the tests were carried out at a pH level where the sperm were inactive, but were readily activated when egg-water at the same pH was added. This pH value was found to be in the neighborhood of 6.0 for the sea water and 7.5 for the isotonic sodium chloride solutions. The actual pH values varied with the individual sea-urchin, and increased if the dry sperm was allowed to age. In these experiments the absolute value is not important, since in every case a control test with egg-water was made along with each test of echinochrome. Accordingly, if echinochrome is the activating agent in egg-water, it should show activating properties at the same pH as the egg-water.

Two methods of testing were employed. In one, the dry sperm, that is, the sperm taken directly from the testis with a minimum of moisture, was diluted to about 1 : 100 in buffered sea water or isotonic NaCl. A drop of this was covered with a cover-glass, and the egg-water and echinochrome pipetted into opposite sides of the drop. In this way the slightest response of the sperm could be detected and a precise comparison made between the two solutions. Adding dry sperm directly to the test solution sometimes gave more spectacular differences in response, but where small differences are involved, the first method is freer from subjective interpretation.

In no case did echinochrome activate the sperm. Each test was accompanied by a test with egg-water at the same pH, in which activation did occur.

Additional tests were carried out at higher pH values to supply more nearly normal conditions for the sperm. The results are not as clear-cut as with inactivated suspensions, since differences in speed of sperm are hard to estimate. However, in no case could it be said that the echinochrome definitely produced an increase in motility greater than did mere dilution with buffered sea water, whereas stimulation by egg-water could usually be seen clearly.

Chemotaxis in Echinochrome

To check the reported chemotactic effect of echinochrome, a few tests were made to compare the migration of sperm up glass capillary tubes. Tubes of the same diameter were washed and filled with sea water, egg-water, and echinochrome solution. The ends were then inserted into buffered sperm suspension, and migration measured at various times. The results were so variable that none of the solutions could be said to be definitely chemotactic on the basis of these few trials. Variations in the alkalinity of the glass probably played some part, since the volume of solution was small in proportion to the surface of the tube, and the buffer capacity of the solutions was low. Under such conditions a shift toward alkalinity could occur and give an illusory chemotactic effect by merely speeding the progress of the sperm.

Activity of the Echinochrome-protein Complex

The complex which echinochrome forms with proteins from the *Arbacia* egg was reported by Kuhn and Wallenfels (1940) to have sperm-stimulating properties in even greater dilutions (1 : 300,000,000,000) than echinochrome alone, in uncombined form. A similar echinochrome complex was extracted from *Arbacia punctulata* by their method. Eggs were frozen, crushed, and extracted with sea water. After filtering, an equal amount of saturated ammonium sulphate solution was added, bringing down a rose-colored precipitate which redissolved in sea water without leaving any residue. It was purified by repeated precipitation, centrifugation, and decantation.

This complex, buffered and tested in the same way as echinochrome, both activated and agglutinated *punctulata* sperm. In biological properties and solubility, therefore, it is the same as the tertiary complex obtained by Kuhn and Wallenfels. In view of the tests with pure echinochrome, however, the activity of the complex would seem to be centered in the protein moiety rather than in the echinochrome.

Separation of the Agglutinating and Activating Properties of Egg-water

To determine more of the nature of the activating agent, egg-water was dialyzed against sea water for a half-hour, then both fractions buffered and tested for activity. The dialysate stimulated without agglutinating, while the residue both stimulated and agglutinated sperm. Dialysis, then, can separate the agglutinating and activating agents. Distillates obtained by gently boiling each fraction also showed activating properties, but the activity disappeared shortly after the distillate was buffered to pH 6. The original, unboiled dialysate retained its activity 24 hours, beyond which it was not tested. Similarly, a distillate from a repeatedly precipitated and washed sample of the echinochrome-protein complex could stimulate, when unbuffered (pH 9.0), and lost its activity within an hour after it was buffered to pH 6.6. This disappearance of stimulating activity from the distillates suggests that the activating agent had been altered during distillation. Improved methods of separation will probably yield a stable stimulating fraction. At present, it is important that distillates of egg-water, of egg-water dialysate, and of the echinochrome complex are similar in that they contain an activating substance.

Discussion

The absence of visible response of *A. punctulata* sperm to echinochrome is in agreement with Tyler's investigations with *Strongylocentrotus purpuratus.* He found that echinochrome brought about no increase in oxygen consumption of the sperm or eggs. On the other hand, these results do not agree with the observations of Hartmann and Schartau on *A. pustulosa,* which was found to be extremely sensitive to echinochrome solutions. The difference in response of *A. pustulosa* and *A. punctulata* could be attributed to species difference, although this would make the similarity of *A. punctulata* to the more distantly related *Strongylocentrotus* appear somewhat anomalous. Another possibility is that Hartmann and Schartau did not control pH in their solutions, since it is not mentioned in any of the papers on *A. pustulosa.* Their results, particularly the activity of highly dilute solutions (1:2,500,000,000), suggest that the activation is due to the normal alkalinity of the sea water used as a solvent.

Whatever the final answer may be with regard to echinochrome, we must search farther for the answer to the general problem of sperm-activation by egg-secretions. Echinochrome is limited in occurrence, even within the class *Echinoidea.* Moreover, the egg-secretions from pigmented eggs will stimulate sperm from unpigmented species, and vice versa (cf. Woodward, 1918, Table I).

In the *Arbacia* egg there is some sperm-activating substance which will dialyze through a collodion membrane. It can, then, be separated from the agglutinating substance with which it is closely associated, but which will not dialyze. However, the echinochrome-protein complex carried with it through seven precipitations the power to activate as well as agglutinate sperm. In view of the ease with which the activator dialyzes, one might well expect it to be washed completely free from the agglutinating substance. Tyler (1939) also reports that partial purification of agglutinin from the keyhole limpet does not free it from activating properties. This leads one to suspect that there may be two substances present which activate sperm: one closely attached to the agglutinating substance, and one easily separated from it. On the other hand, the activating properties of rough distillates from the egg-water dialysate and the partially purified echinochrome-protein complex is some evidence of similarity between the activating agents in both, but it does not prove identity.

Earlier work on extracts from echinoderm eggs offers a possible explanation of these observations. Woodward (1918) obtained, in addition to an ammonium sulphate precipitate of agglutinin, a barium chloride precipitate which showed lipolytic activity. Glaser (1921) pointed out that this lipolysin (and pancreatic lipase as well) could activate sperm. Yet, if this lipase is the activating agent in egg-water, it follows that the activator in the distillates must be some substance other than the lipase, since a protein would not distil. In this respect the work of Clowes and Bachman (1921) takes on added significance. They were not only the first to obtain sperm-activating distillates from egg-water, but also found that higher alcohols (propyl, allyl, and cinnamyl) and related substances activate sperm. Bringing these findings together, one might tentatively suggest that the immediately effective agent in sperm activation is an alcohol freed by the lipase. Then the presence of the alcohol or of the lipase would be adequate to produce sperm activation. It remains to be demonstrated that the sperm-activator that follows the agglutinating fraction is the lipase, and the dialysable, distillable activator is the product of the activity of that lipase. The hypothesis fits the framework of assembled facts, but substantiation will require considerable further investigation of egg-water fractions and egg extracts.

SUMMARY

Echinochrome is not the agent in *A. punctulata* egg-water which stimulates sperm. The echinochrome-protein complex precipitated from the extract of crushed eggs by $(NH_4)_2SO_4$ is an effective sperm stimulator. From egg-water dialysate and from the echinochrome-protein complex a distillate can be obtained which has sperm-activating properties. It is tentatively suggested that a higher alcohol freed by a hydrolytic agent in the egg-water is the stimulating substance acting directly upon the sperm.

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THE NUTRITIONAL REQUIREMENTS OF TRIBOLIUM CONFUSUM DUVAL

I. The Survival of Adult Beetles on Patent Flour and Complete Starvation Diets[1]

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Introduction

The attention of numerous workers has recently been drawn to the problem of determining the nutritional requirements of insects. The problem has been attacked along two general lines of procedure, namely: (1) by observations on the ability of an organism to survive on a given diet; and (2) by analyses of the different regions of the digestive tract of the organism to determine the presence or absence of the various digestive enzymes. The present series of studies on *Tribolium* concerns the former aspect of the problem. The importance of determining the nutritional requirements of *Tribolium* was emphasized in an earlier paper (Schneider, 1940) on thyroid feeding. There it was stated that " When the fields of the nutritional requirements and the endocrinology of insects have been thoroughly worked, we shall be in a much better position to determine the effect of a given vertebrate hormone extract on a given insect than we are at present."

Chapman (1924), Sweetman and Palmer (1928), Street and Palmer (1935), Nelson and Palmer (1935), Bushnell (1938), and Chiu and McCay (1939), all working with *Tribolium,* studied the nutritional requirements with respect to group biology or population growth and maintenance. Since there are many factors in addition to the nutritional quality of the food medium which affect population growth, i.e., population density, cannibalism, and " conditioning " of the food medium (Park, 1934*a*), it seemed expedient to study the problem from a more fundamental viewpoint, namely that of determining the individual performances of isolated beetles fed on various diets. From logical considerations it appears that an approach to the problem from this point of view is likely to lead to a sounder foundation of the nutritional requirements of insects *per se,* than can be attained from following any other line of investigation.

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[2] The writer is greatly indebted to the late Dr. Raymond Pearl for his helpful guidance and criticism during the progress of this investigation, and to Mr. Myron L. Simpson for his willing assistance in the experimental routine.

The present investigation has indicated that the starvation control is of considerable value in nutritional studies, and is particularly desirable in this case in view of the long life (up to three years) of the organism on the so-called " normal diet," flour. The term " normal diet " is here used in the sense that the beetles have been found to grow and reproduce in it, though it may not really be adequate for optimal physiological processes. The value of a particular diet can be determined just as accurately by comparison of the performances of the animals fed on it with those of animals on complete starvation, as it can by comparison with those of animals fed the " normal diet," and in this particular case it is certainly less time-consuming. In other words, if we start with the starvation diet as the zero point on a scale, all other diets, including the present so-called " normal diet " can be referred to the zero point in terms of either positive or negative values, i.e., they will be either better or worse than no food at all.

Statement of the Problem

Pearl and Parker (1924) have stated that ". . . when we study duration of life under normal conditions we are dealing with the combined effects of two variable complexes, inborn organization, on the one hand, and environment, including renewal of available energy and substance by food, on the other hand." The former complex, inborn organization, designated by Ashby (1930) as the " capital " of the organism, has also been termed the inherent vitality of the organism. Since the knowledge of the inherent vitality of *Tribolium* is primarily essential to the knowledge of its general nutritional requirements, the present investigation has been designed to deal principally with this phase of duration of life. Hence, the problem may be stated as that of determining the ability of the adult beetle, *Tribolium confusum* Duval, to survive under conditions of complete starvation. Several more specific problems dealing with the influence of various environmental factors of pre-imaginal life on the ability of the adult beetle to survive conditions of complete starvation are involved.

Technique

Park (1934*b*) has described in detail the general technique for handling *Tribolium* cultures in the laboratory, and there is no need for its repetition here. It is sufficient to state that the beetle spends its entire life history in flour, and by the use of sieves of various sized mesh, the adults, eggs, and all immature forms can be separated from the flour for observation. The sex is determined from the pupal characteristics.

Throughout the present investigation, when eggs were used to start an experiment, a sufficient number was taken from a general stock culture, and allowed to hatch in dishes containing a small quantity of patent flour (Ceresota). The date of hatching was determined within 0.5 day, and the larvae were placed in bottles in a definite quantity of flour (indicated in the experiments). The bottles were kept in a darkened constant temperature incubator at a temperature of 29° C., and a relative humidity of approximately 40 per cent. The food was not changed, and the bottles were not sifted until about the twentieth day after hatching, when it became necessary to sift them once a day to collect pupae. The date of pupation was recorded to the nearest 0.5 day (the color of the pupa indicating whether the pupal stage was reached within the past 0.5 or 1.0 day). The pupae were sexed and placed in sterile vials (1.5 cm. diam. × 2.5 cm. tall) and kept in the incubator under the above-mentioned physical environmental conditions. The date of emergence was recorded to the nearest 0.5 day, and the discarded pupa cases were removed from the vials. The vials were closed with corks covered with cellophane, the corks having a filed groove along the side, and the cellophane being perforated so as to maintain a normal oxygen supply within the vials. The experience of the writer in having beetles escape by eating their way through the cork along the filed groove prompted the use of the cellophane covering. This prevented the boring activity in all but a very few vials where the cellophane was broken, and of course these few cases were immediately discarded. The date of death was recorded to the nearest day, and the period from emergence to death (adult survivorship) was calculated.

When only adults of a known age and sex were needed for an experiment, it was necessary to start by collecting pupae from a general or specific stock culture (indicated in the experiments). These were sexed and placed in vials, from which point the procedure was the same as that described above.

In the one experiment where flour-fed controls were used, the vials were supplied frequently with plenty of fresh flour, so that the organism was always presented with an unlimited quantity of food. Data on the weights of pupae, live and dead imagos, have been reserved for a later publication.

The statistical data are based on frequency distributions of the variables studied. The $\frac{\text{Diff.}}{\text{P. E. Diff.}}$ test for significance was used; any value higher than 3 indicating that the difference was probably not due to sampling errors.

Observations and Results

Survivorship of Adult Beetles, Taken as Pupae from a General Stock Culture and Subjected to Flour and Starvation Diets upon Emergence

In the first experiment 432 pupae (216 of each sex) were isolated from a general laboratory stock culture, and were observed daily for emergence. Since these forms were taken from a general stock culture, they were reared under identical, though unknown factors of larval density. Upon emergence, 100 of each sex were submitted to conditions of complete starvation, and 116 of each sex were given a flour diet. Daily observations were made for mortality, and the time of death was recorded. The time intervening between emergence and death was calculated as adult survivorship. Failure of the organism to respond to agitation with a small camel's hair brush was taken as the criterion for death.

Table I presents the data relative to the survivorship of these two groups of organisms.

Table I

Survivorship of adult beetles on flour and starvation diets.

Group	Range		Mean number days of life at end of 24 days	Median number days of life at end of 24 days	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
Starved adult males....	2.5	21.0	14.235 ±0.274	14.665 ±0.343	4.062 ±0.194	28.53 ±1.46	100
Flour-fed adult males...	2.5	24.0	21.323 ±0.370	24.181 ±0.464	5.908 ±0.262	27.70 ±1.32	116
Starved adult females...	3.0	23.5	13.535 ±0.314	14.000 ±0.394	4.648 ±0.222	34.34 ±1.82	100
Flour-fed adult females.	2.5	24.0	22.964 ±0.240	24.221 ±0.301	3.826 ±0.169	16.16 ±0.76	116
Total starved adults....	2.5	23.5	13.885 ±0.213	14.593 ±0.267	4.459 ±0.150	32.11 ±1.19	200
Total flour-fed adults...	2.5	24.0	22.166 ±0.220	24.202 ±0.276	4.974 ±0.156	22.43 ±0.74	232

The data are presented for the sexes separately and then combined. Since all the beetles on starvation were dead at the end of 24 days, it appeared justifiable to compare their survivorship performances with those of the flour-fed group up to the end of 24 days. This procedure obviated the necessity of observing the flour-fed group for their entire life (which may have been up to three years). For a study of the complete survivorship record of flour-fed *Tribolium,* the reader is referred to Pearl, Park and Miner (1941). In the present case it was not possible to calculate mean length of life of the flour-fed group; instead there is calculated the mean number of days of life lived up to the end of 24 days per beetle exposed to risk.

The difference between the mean length of life of the starved beetles (13.885 ± 0.213 days) and the mean number of days of life at the end

TABLE II

Ungraduated life table for flour-fed and starved adult Tribolium.

Day of age	Flour-fed												Starved											
	Male				Female				Both sexes together				Male				Female				Both sexes together			
	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000
0– 1	116	0	0	1000	116	0	0	1000	232	0	0	1000	100	0	0	1000	100	0	0	1000	200	0	0	1000
1– 2	116	0	0	1000	116	0	0	1000	232	0	0	1000	100	0	0	1000	100	0	0	1000	200	0	0	1000
2– 3	116	3	26	1000	116	0	0	1000	232	3	13	1000	100	3	30	1000	100	1	10	1000	200	4	20	1000
3– 4	113	2	18	974	116	1	9	1000	229	3	13	987	97	1	11	970	99	3	30	990	196	4	20	980
4– 5	111	0	0	957	115	2	17	991	226	2	9	974	96	2	21	960	96	3	31	960	192	5	26	960
5– 6	111	0	0	957	113	0	0	974	224	0	0	966	94	1	11	940	93	5	54	930	187	6	32	935
6– 7	111	2	18	957	113	0	0	974	224	2	9	966	93	2	22	930	88	3	34	880	181	5	28	905
7– 8	109	4	37	940	113	0	0	974	222	4	18	957	91	1	11	910	85	3	35	850	176	4	23	880
8– 9	105	2	19	905	113	2	18	974	218	4	19	940	90	4	44	900	82	1	12	820	172	5	29	860
9–10	103	0	0	888	111	0	0	956	214	0	0	922	86	2	23	860	81	6	74	810	167	8	48	835
10–11	103	0	0	888	111	0	0	956	214	0	0	922	84	1	12	840	75	5	67	750	159	6	38	795
11–12	103	1	10	888	111	0	0	956	214	1	5	922	83	2	24	830	70	3	43	700	153	5	33	765
12–13	102	1	10	879	111	0	0	956	213	1	5	918	81	6	74	810	67	9	134	670	148	15	101	740
13–14	101	1	10	871	111	0	0	956	212	1	5	913	75	12	160	750	58	8	138	580	133	20	150	665
14–15	100	1	10	862	111	0	0	956	211	1	5	909	63	21	333	630	50	8	160	500	113	29	257	565
15–16	99	0	0	853	111	0	0	956	210	0	0	905	42	12	286	420	42	10	238	420	84	22	262	420
16–17	99	1	10	853	111	0	0	956	210	1	5	905	30	12	400	300	32	10	303	320	62	22	355	310
17–18	98	2	20	845	111	2	18	956	209	4	19	901	18	5	278	180	22	5	227	220	40	10	400	200
18–19	96	1	10	827	109	1	9	939	205	2	10	884	13	6	462	130	17	8	471	170	30	14	466	150
19–20	95	2	21	819	108	1	9	931	203	3	15	875	7	4	571	70	9	4	444	90	16	8	500	80
20–21	93	0	0	802	107	2	19	922	200	2	10	862	3	3	1000	30	5	1	200	50	8	4	500	40
21–22	93	0	0	802	105	0	0	905	198	0	0	853	0	0	—	0	4	3	750	40	4	3	750	20
22–23	93	1	9	802	105	1	10	905	198	2	10	853					1	1	1000	10	1	1	1000	5
23–24	92	1	9	793	104	0	0	896	196	1	5	845					0	0	—	0	0	0	—	0
24–25	91	0	0	784	104	0	0	896	195	0	0	841												

of 24 days of the flour-fed group (22.166 ± 0.220 days) is 8.281 ± 0.306 days (27.06 × P. E.). The sex difference, though not a significant one in the case of the starved forms, indicates that the males on the average survived slightly longer than the females. The opposite is true for the flour-fed group, i.e., the females showed a higher mean number of days of life at the end of 24 days than the males. The latter difference amounts to 1.641 ± 0.441 days (3.72 × P. E.).

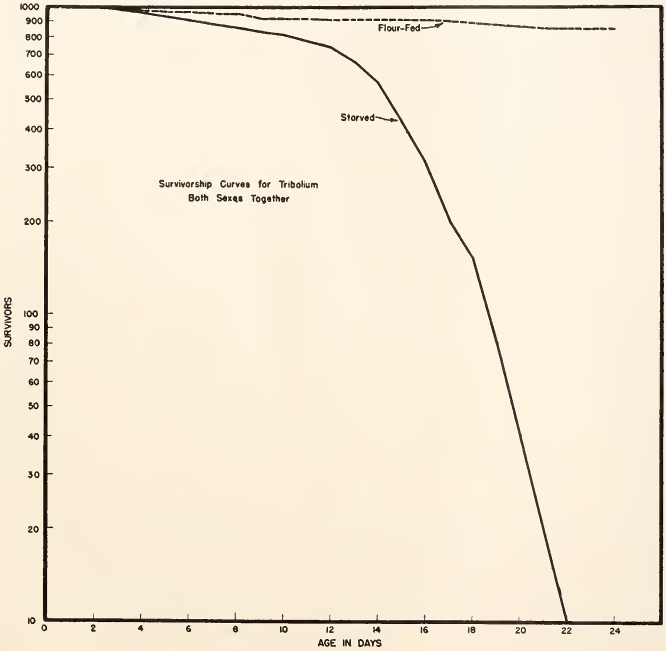

FIG. 1. Survivorship curves for the flour-fed and starved adults of *Tribolium*, taken as pupae from a general stock culture. Flour-fed: $N = 232$; observed 24 days. Starved: $N = 200$; observed entire life.

Table II is the ungraduated life table for these two groups of organisms. The four columns under each sex heading show: (1) the absolute number of beetles living at the beginning of each day of age and therefore exposed to the risk of dying during that day; (2) the number of deaths actually occurring during each day of age; (3) death rate for each day of age per 1000 living at the beginning of the day; and (4) survivors per 1000 exposed to risk at the beginning of each day of age.

It is true that a few females of the starved group lived longer than any males of that group, but the fact that a greater number of males survived through the 12- to 14-day period accounts for the higher average survivorship of the males. In the flour-fed group the females took an early lead in survivorship and maintained that lead at an increasing rate to the end of the 24-day period of observation. Figure 1 shows the survivorship curves for the starved and flour-fed groups, sexes combined.

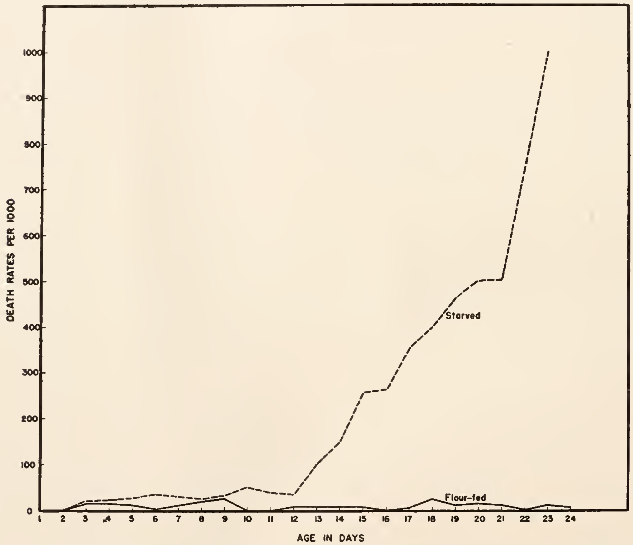

Fig. 2. Observed (ungraduated) death rates (deaths per 1000 exposed to risk) for flour-fed and starved adult *Tribolium*, taken as pupae from a general stock culture.

The only other factor of importance in discussing this particular experiment is the mortality rate. This may be studied from the life table for these two groups of beetles (Table II) and from the mortality curves presented in Fig. 2.

From Fig. 2 it is evident that the mortality rates exhibited no great differences between the flour-fed and starved groups of organisms up to the twelfth day, though during this time the rate for the starved group was slightly higher, on the whole, than that for the flour-fed group. After the twelfth day the mortality rate of the starved beetles went up very rapidly, while that of the flour-fed group continued to fluctuate just a little above the zero line. The point of interest here is that starving the adult beetle for the first 10 or 12 days after emergence does not seriously affect the rate of mortality. If starvation is continued beyond this point, however, the necessity for an exogenous supply of matter and energy for the maintenance of normal physiological processes manifests itself in a sudden rise in the rate of mortality. Whether or not the adult beetle will recover from the effects of starvation if returned to a diet of flour on the twelfth day is a problem for further investigation.

An observation worthy of note here is that the starved beetles produced fecal pellets throughout their entire lives.

The question may be raised as to whether the starved female adults may not have laid eggs and eaten them, and hence may not represent a completely starved group. To answer this point, it can first be stated with certainty that no eggs were ever seen in the vials containing the starved females. This means that the females either ate the eggs immediately after laying them, or that they never laid any eggs at all. Further experimental evidence points to the latter conclusion as the correct one. Virgin females from the flour-fed group were observed carefully for daily egg-laying. In no case were any eggs found under 12 days. At the end of 20 days the cumulative egg-laying record of 185 virgin females in flour was as follows:

49 females had laid 0 eggs at the end of 20 days.
29 females had laid 1 egg at the end of 20 days.
22 females had laid 2 eggs at the end of 20 days.
20 females had laid 3 eggs at the end of 20 days.
21 females had laid 4 eggs at the end of 20 days.
8 females had laid 5 eggs at the end of 20 days.
6 females had laid 6 eggs at the end of 20 days.
13 females had laid 7 eggs at the end of 20 days.
4 females had laid 8 eggs at the end of 20 days.
2 females had laid 9 eggs at the end of 20 days.
3 females had laid 10 eggs at the end of 20 days.
2 females had laid 11 eggs at the end of 20 days.
3 females had laid 12 eggs at the end of 20 days.

It is clear that over 76 per cent of these beetles had laid fewer than 5 eggs each at the end of 20 days.

From this sample of 185 virgin, flour-fed females, it is evident that in the absence of the male, the beginning of egg-laying is decidedly retarded (mated, flour-fed females begin laying 2 or 3 days after emergence). This fact, as well as the low mean length of life of starved females, in addition to the poor body nutrition of the starved group at the time when they would have started laying, all point strongly to the probability that the starved females never laid any eggs during their lives. Furthermore, the fact that the mean length of life of the starved females was slightly less than that of the starved males indicates that even if the females did lay a few eggs and eat them, this action did not contribute greatly to their supply of nutrition.

TABLE III

Length of larval period for various larval population densities.

Larval density	Range		Mean length of larval period (days)	Median length of larval period (days)	Standard deviation (days)	Coefficient of variation (per cent)	N
	Min. (days)	Max. (days)					
(*a*) 100 larvae per 100 grams flour......	25.5	40.0	34.095 ±0.106	33.431 ±0.133	2.719 ±0.075	7.97 ±0.22	297
(*b*) 10 larvae per 10 grams flour......	25.5	52.0	35.922 ±0.149	35.281 ±0.187	3.726 ±0.105	10.37 ±0.29	285
(*c*) 300 larvae per 100 grams flour......	29.5	64.0	37.460 ±0.229	36.038 ±0.287	5.642 ±0.162	15.06 ±0.44	276

It is evident that when an experiment such as this is started with pupae from a general stock culture, nothing is known of the population factors under which the larvae developed. If pupae are taken from various stock cultures where larval density factors are almost certain to be unequal, and if larval density does have an influence on adult inherent vitality, then it is clear that the experiment has not been well controlled. In the present experiment, the pupae were all taken from the same stock culture, and hence developed as larvae under identical conditions, though these conditions were probably not optimal. In order to test the influence of larval density on adult inherent vitality, and to find the larval density conducive to the greatest adult vitality, further experiments were made.

The Influence of Larval Density on Inherent Vitality of Adult Tribolium

Larval Development.—For this investigation, eggs were collected from a general laboratory stock culture of *Tribolium,* and allowed to hatch in a small quantity of flour. Every 12 hours the newly-hatched larvae were collected, and placed in various sized bottles under three different food-density relationships. Each group contained 300 larvae and the density relationships were as follows:

(*a*) 100 larvae in 100 grams of flour.
(*b*) 10 larvae in 10 grams of flour.
(*c*) 300 larvae in 100 grams of flour.

The larvae in (*b*) were kept in 1-ounce bottles; those in (*a*) and (*c*) in half-pint milk bottles. Not only were the survivorship performances of the resulting adults compared for the three series, but also the developmental periods for the larvae.

Table III presents data relative to the length of time spent in the larval stage for the three larval population densities.

From Table III it is evident that the larvae in density (*a*) developed in less time than those from either (*b*) or (*c*). Comparing the mean length of larval period for group (*a*) with that of group (*b*) and (*c*), respectively, we find differences of 1.827 ± 0.183 days (9.98 × P. E.) and 3.365 ± 0.252 days (13.35 × P. E.). Each difference is a statistically significant one, and each points to a more rapid larval development of the (*a*) group. Comparing (*b*) and (*c*), the difference of 1.538 ± 0.273 days (5.63 × P. E.) is also a statistically significant one, and indicates a more rapid rate of development for the (*b*) than for the (*c*) group. From the standpoint of larval development, density (*a*) produced the most rapid rate; density (*b*) the next most rapid rate; and density (*c*) the slowest rate. These observations are substantially in accord with those of Park (1938). The standard deviations indicate that the variation in length of larval period is positively associated with the absolute length of larval period; i.e., with an increase in mean length of larval period comes an increase in the amount of variation. This fact may be grasped more quickly by reference to Fig. 3.

The curve representing density (*a*) is high and narrow; that for density (*c*) is low and broad, tailing off far to the right; while that for density (*b*) falls between these two extremes. Not only is the absolute variation greater for the greater length of larval period, but also the variation relative to the mean (coefficient of variation) as indicated in Table III.

Of the 300 larvae which were started in each series 297 reached pupation in density (*a*), 285 in density (*b*), and 276 in density (*c*), indicating that the larval mortality was influenced by the density factor also. The percentage mortality for the three groups was as follows: (*a*) 1.00 ± 0.57 per cent; (*b*) 5.00 ± 1.26 per cent; and (*c*) 8.00 ±

1.57 per cent. Comparing these percentages we find the following differences: (*a*) vs. (*b*), 4.00 ± 1.38 per cent (2.90 × P. E.); (*a*) vs. (*c*), 7.00 ± 1.67 per cent; (4.19 × P. E.); and (*b*) vs. (*c*), 3.00 ± 2.01 per cent (1.49 × P. E.). Only one of these differences is statistically significant; that existing between groups (*a*) and (*c*). Clearly something in addition to chance fluctuation caused the heavier mortality in group (*c*). In all probability, it was the larval density factor which was responsible for this effect. The difference between (*a*) and (*b*) (2.90 × P. E.) is a borderline case of significance, and hence cannot be considered a real difference. The difference between (*b*) and (*c*) is clearly not significant. These observations on the density factor as related to larval mortality are also in accord with those of Park (1938).

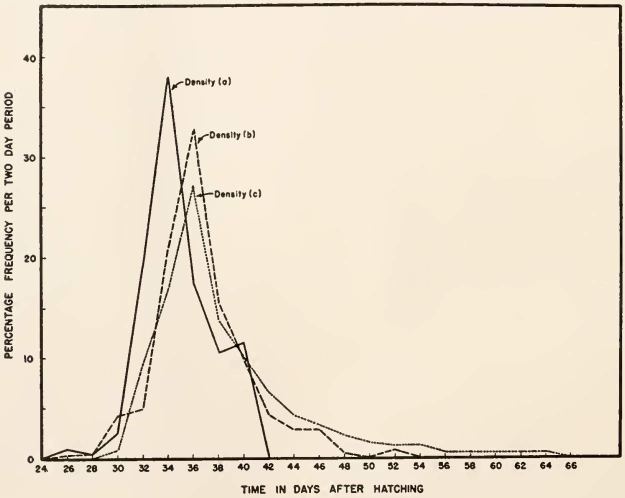

FIG. 3. Frequency distribution of length of larval period for various larval population densities.

It is easy to understand the differences that exist between densities (*a*) and (*c*), since there are three times as many larvae per unit of environment in (*c*) as there are in (*a*). However, the differences between (*a*) and (*b*) are more difficult to fathom in view of the fact that the two had the same population density per unit of environment. The only difference lay in the size of the total populations; one was made up of 10 larvae in 10 grams of flour, the other of 100 larvae in 100 grams of flour. Just why the 100/100 condition was conducive to a lower larval mortality, and more rapid larval development than the 10/10 is not apparent at the moment, though the phenomenon is certainly worthy of further investigation.

Adult Survivorship.—After reaching the adult stage, the organisms from the three larval densities described above were isolated and subjected to complete starvation. The survivorship (period from emergence to death) data for the sexes separately, then combined for the three experimental groups are presented in Table IV.

There was only a small and statistically insignificant difference in survivorship between the sexes in densities (*a*) and (*c*). In density (*b*) this difference is 1.002 ± 0.170 days (5.88 × P. E.). In all three cases, however, the differences indicate that the females survived longer than the males. For the combined sexes, there are large differences in survivorship between the adults reared as larvae, under different population densities. Group (*a*) showed the best survivorship; group (*c*) the next best; and (*b*) the worst. A comparison of the mean length of adult life of group (*a*) with that of group (*b*) and (*c*) respectively, reveals differences of 2.349 ± 0.166 days (14.15 × P. E.) and 1.582 ± 0.172 days (9.20 × P. E.). In each case group (*a*) exhibited a superiority in survivorship which is far outside the limits of chance fluctuation. A comparison of mean survivorship of groups (*b*) and (*c*)

TABLE IV

Length of life of starved adults, reared under various larval population densities.

Larval density	Range		Mean length of life (days)	Median length of life (days)	Standard deviation (days)	Coefficient of variation (per cent)	N
	Min. (days)	Max. (days)					
Male							
(*a*) 100 larvae per 100 grams flour	1.5	25.0	18.739 ±0.118	19.353 ±0.148	2.086 ±0.083	11.13 ±0.45	142
(*b*) 10 larvae per 10 grams flour	3.0	23.0	16.086 ±0.159	16.500 ±0.199	2.887 ±0.112	17.94 ±0.72	150
(*c*) 300 larvae per 100 grams flour	3.5	21.5	17.007 ±0.154	17.568 ±0.193	2.632 ±0.109	15.47 ±0.65	133
Female							
(*a*) 100 larvae per 100 grams flour	3.0	24.0	18.753 ±0.173	19.375 ±0.217	3.169 ±0.122	16.89 ±0.67	152
(*b*) 10 larvae per 10 grams flour	8.0	23.5	17.088 ±0.061	17.202 ±0.076	1.018 ±0.043	5.96 ±0.26	125
(*c*) 300 larvae per 100 grams flour	4.0	22.0	17.547 ±0.169	18.333 ±0.212	2.918 ±0.119	16.62 ±0.70	136
Total							
(*a*) 100 larvae per 100 grams flour	1.5	25.0	18.763 ±0.128	19.393 ±0.160	3.251 ±0.090	17.32 ±0.49	294
(*b*) 10 larvae per 10 grams flour	3.0	23.5	16.414 ±0.105	17.046 ±0.131	2.593 ±0.074	15.79 ±0.46	275
(*c*) 300 larvae per 100 grams flour	3.5	22.0	17.181 ±0.115	18.032 ±0.144	2.794 ±0.081	16.26 ±0.48	269

brings to light a difference of 0.767 ± 0.156 days (4.91 × P. E.), which difference is also a statistically significant one. In this case, group (*c*) possessed the greater survivorship value. These differences are illustrated by the survivorship curves in Fig. 4.

The influence of larval density on adult survivorship is clear, but the exact differential effect of the different densities is not so apparent.

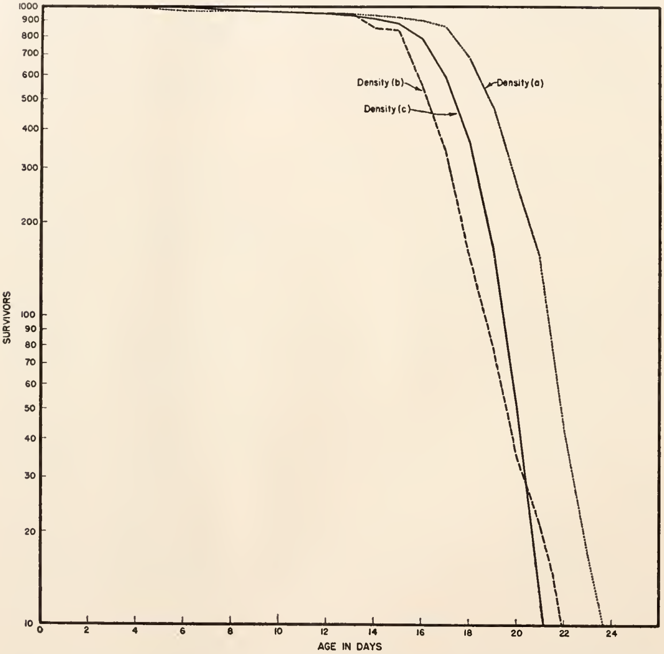

FIG. 4. Survivorship curves of starved adult *Tribolium* reared in various larval population densities.

It is true that group (*a*) which showed the most rapid larval development also exhibited the longest record of survivorship. But group (*b*), which showed the second most rapid larval development, survived the shortest period of time on starvation; and, conversely, group (*c*), which showed the slowest larval development, possessed the second best adult viability. These results indicate the desirability of further work to determine the differential effects on growth, development, and inherent vitality of various sized total populations kept at a constant density per unit of environment.

This experiment has demonstrated that larval density is a factor which must be controlled in investigations on adult inherent vitality. From this it follows that the "capital" of the newly emerged adult *Tribolium* is not solely dependent upon the inborn organization of the organism, but also upon the environmental influences which have been effective throughout its immature stages. In a study such as that of Greiff (1940), where the length of life of isolated adults of *Drosophila melanogaster* and that of its mutant, Ebony, under conditions of complete starvation were compared, and where it was admitted that ". . . the ebony mutant fly was observed to do better under laboratory conditions than the wild-type fly" (i.e., it produced higher larval densities in the stock bottles from which the flies were isolated), it would obviously have been desirable to control the larval density factor.

TABLE V

Length of larval period for descendants of parents of indicated ages.

Age of parents	Range		Mean length of larval period (days)	Median length of larval period (days)	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
1 month	30.5	54.0	37.516±0.154	37.416±0.193	3.830±0.109	10.21±0.29	279
6 months	25.5	48.0	33.896±0.175	33.618±0.219	4.235±0.124	12.49±0.37	265

To separate the environmental factor of larval density from the inborn organization variable, and to test the relationship between age of parent and inherent vitality of offspring, further experiments were made.

Influence of Age of Parents on Vitality of Adult Offspring

Larval Development.—Eggs from two different stocks, one a month old, the other six months old, were collected and kept separate for hatching, whereupon 300 larvae from each group were placed in flour in half-pint milk bottles at a density of 100 larvae to 100 grams of food. Every factor in the experimental procedure was held constant except that of the age of the parent generations.

The data on larval development of these two groups of organisms descended from parents of different ages are set out in Table V.

The mean figures for length of larval period for the two groups reveal a difference of 3.620 ± 0.233 days (15.53 × P. E.). The progeny of the six-months-old parents developed at a rate significantly more rapid than those of parents one month old. The frequency distribution curves in Fig. 5 illustrate this point clearly.

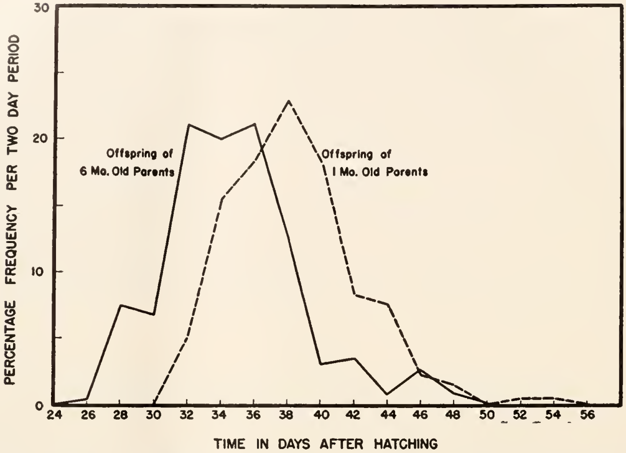

Fig. 5. Frequency distribution of length of larval period of beetles descended from stocks of various ages.

It is apparent that the six-months-old parents, having reached a more highly productive and more mature period of life, produced progeny which developed more rapidly than those produced by the younger, less productive, one-month-old parents. The variability in length of larval period for the two groups of progeny exhibited no striking difference. The larval mortality for the progeny of one-month- and six-months-old

Table VI

Length of life of starved adults descended from parents of indicated ages.

Age of parents	Range		Mean length of life (days)	Median length of life (days)	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
Male							
1 month	11.0	21.5	16.196 ±0.136	16.160 ±0.170	1.992 ±0.096	12.30 ±0.60	97
6 months	6.0	22.5	17.450 ±0.136	17.791 ±0.170	2.129 ±0.096	12.20 ±0.56	111
Female							
1 month	4.0	26.0	16.697 ±0.177	17.000 ±0.222	2.684 ±0.125	16.07 ±0.77	104
6 months	2.5	24.5	18.005 ±0.174	18.555 ±0.218	2.443 ±0.123	13.57 ±0.69	90
Total							
1 month	4.0	26.0	16.455 ±0.124	16.716 ±0.155	2.618 ±0.088	15.91 ±0.55	201
6 months	2.5	24.5	17.701 ±0.112	18.019 ±0.140	2.345 ±0.079	13.25 ±0.45	201

parents respectively, was 3.67 ± 1.08 per cent and 11.67 ± 1.85 per cent. The difference here is 8.00 ± 2.14 per cent (3.74 × P. E.) and indicates a significantly greater amount of mortality in the progeny of the six-months-old parents than for those of one-months-old parents.

Survivorship of Adults.—Upon emergence, 201 adults from each group were subjected to complete starvation. The numerical data on length of life of starved adults descended from parents one and six months old respectively are gathered into Table VI.

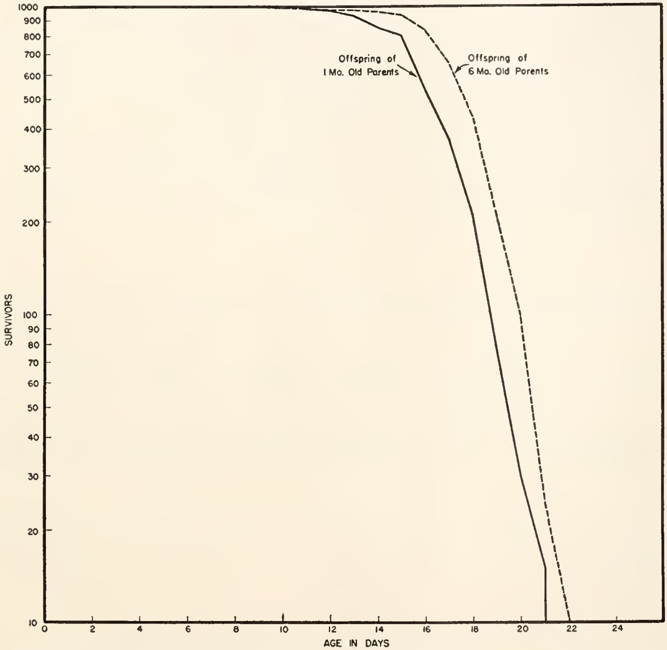

FIG. 6. Survivorship of starved adult beetles descended from parents of indicated ages. $N = 201$ in each group.

The sex difference in survivorship of the two groups is not large, though the females again showed a slight superiority. When the survivorship of the two groups is compared for either females, males, or for the combined sexes, there appear large differences. For the females, the difference is 1.408 ± 0.248 days (5.68 × P. E.); for the males it is

1.254 ± 0.192 days (6.52 × P. E.); and for the sexes together it is 1.246 ± 0.167 days (7.46 × P. E.). All of these differences are highly significant and in each case point to a longer survivorship for the progeny of 6-months-old parents than for those of one-month-old parents. The difference in survivorship of the two groups (sexes combined) is illustrated in Fig. 6.

The sex ratios of the two groups, though not significantly different from each other, or from a 50–50 ratio, are of interest in view of the fact that the parents which produced the most rapidly developing larvae and the most viable adults, also produced the highest proportion of male offspring. This observation is essentially in accord with that of Lawrence (1940) on *Drosophila melanogaster.*

Evidently parental age is a factor which must be controlled carefully in experiments on inherent vitality. It follows that parent age must be considered in determining the basic foundation of our knowledge of the nutritional requirements of *Tribolium.*

TABLE VII

Survivorship of adult Tribolium subjected, at the indicated ages, to a starvation diet.

Starvation begun	Range		Mean length of life after starvation (days)	Median length of life after starvation (days)	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
(*a*) At emergence...	4.0	26.0	16.455 ±0.124	16.716 ±0.155	2.618 ±0.088	15.91 ±0.55	201
(*b*) At age 20 days .	2.5	26.5	16.841 ±0.107	17.000 ±0.134	3.390 ±0.075	20.13 ±0.46	456
(*c*) At age 125 days	3.0	20.0	11.676 ±0.174	11.687 ±0.218	2.892 ±0.123	24.76 ±1.39	125
(*d*) At age 220 days	4.5	14.5	9.625 ±0.221	9.250 ±0.277	1.937 ±0.156	20.12 ±1.68	35

In order to determine the influence of the age of the adult beetle on its ability to survive conditions of complete starvation, a final experiment was performed.

Influence of Age on Inherent Vitality of Adults

Isolated adults fed a flour diet from the time of emergence to the time of starvation made up three of the four series of organisms in this experiment. Starvation was begun at ages 20 days, 125 days, and 220 days respectively in these three groups. Another group representing the controls were starved from the day of emergence. The beetles in all four series were descended from parents of equal age (six months) and were reared under identical larval densities (100 larvae in 100 grams of flour). The three groups of organisms that were fed for a while and then starved represent select groups, since only those remaining alive at the desired time were used. The factor of selection does not impair the value of the experimental data, however, since we are not interested in the viability of those that died before a particular age, but only in the ability of those living at a certain age to survive when transferred to a starvation diet.

The data relative to the survivorship of these four groups of organisms are presented in Table VII.

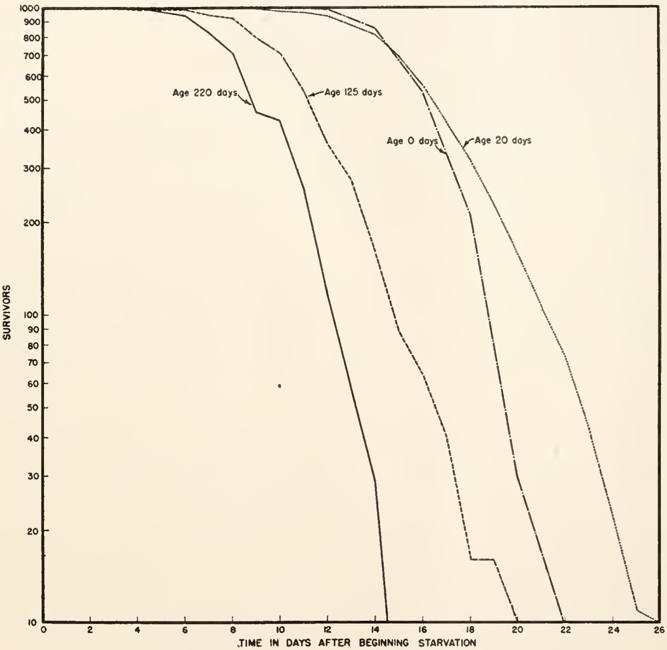

FIG. 7. Survivorship of adult *Tribolium* placed upon a starvation diet at various ages.

From Table VII it is evident that there exist significant differences between each two mean values except in the case of groups (*a*) and (*b*), which is only 0.396 ± 0.164 days (2.41 × P. E.). This difference, though statistically not certainly significant, points to a better survivorship of 20-day-old adults than newly emerged adults when both are subjected to starvation. The beetles of the two higher age groups all survived a shorter period of time on starvation than did those of the two younger groups. The influence of age on the ability of adults to survive under conditions of starvation is illustrated graphically in Fig. 7.

The results suggest that the age at which the beetle survives best under starvation is something more than 0 and less than 100 days. The optimal point is probably in the neighborhood of 20 days. Investigations are now in progress to determine the exact age at which the longest survivorship under starvation occurs.

SUMMARY

The experimental data set forth in this paper have served to show that:

1. Isolated adults of *Tribolium confusum* Duval, subjected to conditions of complete starvation upon emergence, or shortly thereafter, will survive up to 26.5 days depending upon the conditions of the experiment.
2. Survivorship of starved adults is shortened and the period of larval development is lengthened by an increase in larval population density.
3. Survivorship of starved adults is significantly longer and the period of larval development is significantly shorter for progeny of 6-months-old parents than for those of one-month-old parents.
4. Survivorship of starved adults is shortened with increasing age.

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THE PITUITARY REGULATION OF MELANOPHORES IN THE RATTLESNAKE

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Introduction

Only recently has the pituitary been linked with certain color changes in reptiles (Noble and Bradley, 1933; Kleinholz, 1935, 1938*a, b*; Parker, 1938). All of these observations are concerned with the lizard metachrosis and indicate that the pars intermedia of the pituitary gland plays an important rôle in the regulation of the melanophores. The present study reveals that a similar concept must be extended to the snake, since in this animal the dispersed phase of the melanophores is likewise dependent upon the pituitary secretion. Furthermore, certain aspects of the melanophores' arrangement and activity of the snake offer an interesting contrast to those described for the chromatophores of the lizard.

Material and Methods

This study deals primarily with the observations of the effect of hypophysectomies and subsequent replacement injections on the activity of the skin melanophores of the prairie rattlesnake, *Crotalus v. viridis* Raf. These specimens were collected in the eastern part of Wyoming. Similar experiments were later extended to several other species of snakes.

The operative removal of the snake pituitary is relatively simple, since the lower jaw can be retracted sufficiently to expose the entire roof of the oral cavity. Intraperitoneal injections of 10 per cent Nembutal served for anesthesia as recommended by Clark (1937). After the skin and muscles have been removed over the basi-sphenoid region the more exact site of the gland can usually be seen through the semi-transparent bone. The latter is chiseled away and then the whole gland may easily be removed. After this operation the bone capsule is replaced and the skin stitched. A strictly aseptic technique is not necessary.

The pars intermedia is very pale and may easily be distinguished from the highly vascular and pink pars anterior. Since the intermedia tissue in the rattlesnake is approximately one-third to one-fourth the size of the anterior lobe and rather loosely attached to the latter, it may be removed independently and completely.

The effect of intermedin (melanophore-dispersing hormone) on the operated animals was studied by means of intraperitoneal injections of an extract of the pars anterior of the chicken pituitary. This tissue contains an unusually high concentration of melanophore-dispersing hormone and was prepared and assayed according to the method of Kleinholz and Rahn (1939, 1940).

The effect of these various operations upon the ophidian melanophore could most easily be observed and studied in the living, anesthetized animal with a dissecting microscope, since the branches of these large chromatophores extend into the epidermal layer and are contrasted sharply with the homogeneous yellow background of the dermis. For permanent recordings of the various changes scales were clipped, fixed in alcohol and prepared as whole mounts. To supplement this series other scales were serially sectioned at 10 micra.

These operations yielded very striking results in the rattlesnake and led to similar experiments in other species of snakes. Although their gross color changes were not as striking, the melanophores responded in a similar manner to both hypophysectomies and injections. Altogether seven rattlesnakes were hypophysectomized and several of these animals were observed for four months after the operation. The other operated snakes included five garter snakes, *Thamnophis ordinoides;* one ribbon snake, *Thamnophis radix* (B. and G.); one bull snake, *Pituophis s. sayi* (Schlegel); and four Florida water snakes, *Natrix sipedon pictiventris* (Cope).

Observations

The background color of the prairie rattlesnake is a homogeneous light yellow color covered by various melanophores and pigmented epidermal cells whose groupings and concentrations form various light- and dark-brown scales. The latter are responsible for the typical color pattern of the rattlesnake (Fig. 2). Before describing the induced color changes, it seems desirable to consider first the histology of the rattlesnake skin, since the arrangement and activity of its melanophores do not correspond in all respects with those described for the lizards (von Geldern, 1921; Schmidt, 1917; et al.).

Epidermis

One of the outstanding features of this tissue is the occurrence of typical, branched melanophores and their processes which in the lizard seem to be confined almost entirely to the dermis. The epidermis is divided conveniently into two layers, the stratum germinativum and the stratum corneum.

The cells of the germinativum are cuboidal to squamous and arranged in two or three rows. Among these cells one finds the cell body and branches of a small melanophore which will be referred to as the epidermal melanophore (Figs. 1, 5), although the cell body may often lie just below the basement membrane. Whenever it appears in the epidermis, it is probably a migrant from below. One must recognize two varieties of this rather evenly branched epidermal melanophore. One is large (200 micra) and found chiefly on the dorsal side where it contributes to the color of the dark-brown scales (Fig. 5). The other epidermal melanophore is much smaller (130 micra) and confined chiefly to the lateral scales. This cell is more delicate in structure and contains relatively little melanin (Fig. 9).

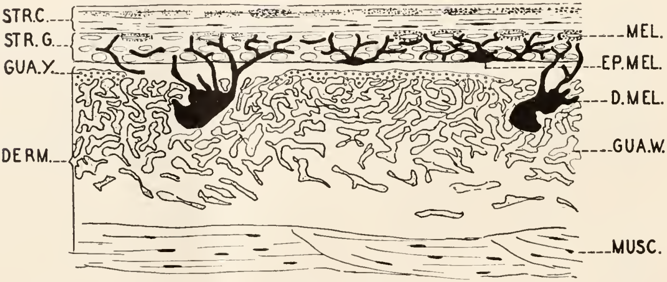


FIG. 1. Schematized section through a scale of the rattlesnake. The melanin deposits, *MEL.*, of the stratum corneum, *STR.C.*, are laid down first in the stratum germinativum, *STR.G.*, near the branches of the epidermal melanophore, *EP.MEL.*, and the dermal melanophore, *D.MEL.* The dermis, *DERM.*, contains a thick layer of white-reflecting guanophores, *GUA.W.*, covered by a thin layer of yellow-reflecting guanophores, *GUA.Y.* Muscle is denoted by *MUSC.*

The dermal melanophore (Figs. 1, 7) is discussed below. Yet, it must be briefly considered here as part of the epidermis, since its branches are found among the epidermal cells, while its cell body is always restricted to the dermis.

In the upper layer of the stratum germinativum the epidermal cells begin to keratinize and it is here that the first signs of intra and some inter-cellular melanin granules appear. Above this region is found the stratum corneum where the cells are completely keratinized and flattened. This region is destined to be cast at the next shedding. It has long been observed in older studies that this layer, the shedding skin, contains melanin granules which correspond in their horizontal distribution to the pigmented areas below. Whether or not these pigmented epidermal cells (Figs. 1, 6) represent independent melanin producers is still questionable. At least they contribute greatly to the color intensity of the dark dorsal scales where they are especially prominent. However, they are not limited to this region alone, since they appear wherever melanophores occur and thus suggest a dependence on these cells.

Dermis

In this region are found the largest melanophores, the dermal melanophores (Figs. 1, 7) whose branches terminate among the epidermal cells. Their cell bodies lie imbedded among the dermal chromatophores responsible for the white-yellow background color of these animals. The latter pigment cells never extend into the epidermis. Their ramifying processes are filled with very small, alcohol-resistant crystals which reflect a white light and are doubly refractive under the polarizing microscope (*GUA.W.,* Fig. 1). Thus these cells may be regarded as guanophores according to the classification of Schmidt (1917). This white-reflecting guanophore stratum is in most places covered by a very thin layer of yellow-reflecting guanophores (*GUA.Y.,* Fig. 1) responsible for the almost homogeneous yellow background color exhibited by the scales. In a few places, this last-mentioned layer is absent (Fig. 1), such as the white-tipped dorsal scales and the regions directly above each dermal melanophore. In these places the dermis reflects only white color.

Effect of Hypophysectomy

The total removal of the pituitary as well as the extirpation of the pars intermedia alone causes a complete pigment concentration in all melanophore types. This would indicate that the pars intermedia is the only pituitary tissue responsible for the normally dispersed state of these cells. Evidence of a successful removal of this gland may be observed one hour post-operatively. Both types of melanophores begin to concentrate their pigment and the long branches of the dermal chromatophores give the impression of disappearing below the epidermis leaving small white islands in the otherwise yellow-reflecting guanophore area. After 4 to 24 hours the concentration is complete. The dermal melanophore pigment is clumped in an irregular fashion (Fig. 8). Granules of the guanophores do not seem to be affected.

It must be pointed out, however, that the snake as a whole does not necessarily appear lighter after the operation. This paling awaits the shedding of the stratum corneum, since this layer contains an abundance of already deposited melanin which may obscure any changes in the melanophores themselves. As soon as shedding has occurred, usually three to four weeks after the operation, a striking change is observed (Fig. 2). (For similar observations on the induction of shedding in the snake after removal of the pituitary, see Schaefer (1933).) This paling is permanent, since little, if any, pigment is deposited hereafter.

Injection of Intermedin Hormone

The intraperitoneal injections of intermedin into an hypophysectomized animal cause complete dispersion in all melanophores. At least one hour is necessary before the first effects can be seen, yet 12 hours or more are required for the completion of this process (Figs. 4, 9). Large doses (several thousand *Anolis* units) will maintain complete dispersion for several days. The dermal melanophore dispersion, however, seems to be maintained by a lower intermedin concentration, since it may remain in this phase for several days after the epidermal melanophores have already completely concentrated their pigment.

It is interesting to observe that an hypophysectomized rattlesnake which has attained maximal pigment dispersion resulting from an intermedin injection is notably lighter than a normal animal (Figs. 3, 4). This emphasizes again that much of the color intensity of this animal is due to the melanin deposits in the keratinized portion of the epidermis and not entirely to the state of melanin dispersion in the chromatophores.

PLATE I

EXPLANATION OF FIGURES

2. Two normal (dark) and two hypophysectomized (light) prairie rattlesnakes. The control animal (*x*) has just shed and appears lighter than the other control animal which is about ready to cast its skin.

3. An hypophysectomized rattlesnake before the injection of intermedin hormone (anesthetized animal).

4. Same animal as in Fig. 3 photographed 24 hours after the injection of intermedin hormone. Notice that in spite of maximal pigment dispersion it is still lighter than the normal control animal in Fig. 2. (For explanation see text.)

FIGS. 5–9. Photomicrographs taken from whole mounts of various scales. × 140.

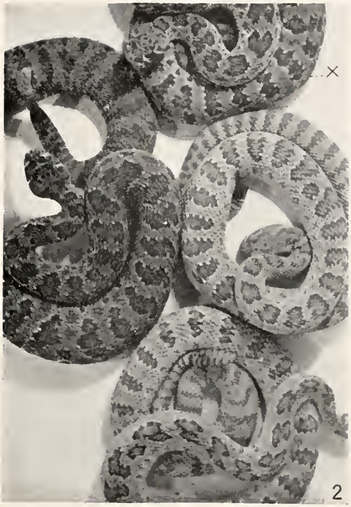
5. The large variety of epidermal melanophore which occurs primarily in the dark dorsal scales; maximal pigment dispersion.

6. Melanin containing epidermal cells (*m.*) from a dark, dorsal scale surrounded by epidermal melanophores (*ep. m.*) whose pigment is concentrated.

7. Dermal melanophores from a lateral scale, notice the bush-like appearance, finer branches, and lack of definite pattern.

8. Lateral scale of an hypophysectomized animal showing pigment concentration in the small variety of epidermal melanophore (*ep. m.*) and in the dermal melanophore (*d. m.*).

9. Lateral scale from same animal as Fig. 8; 6 hours after intermedin injection. Notice partial dispersion of pigment in both types of melanophores.



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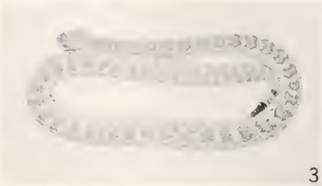

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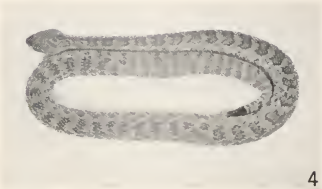

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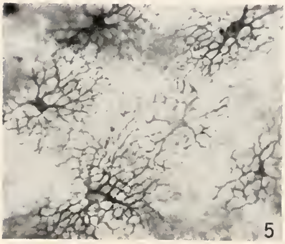

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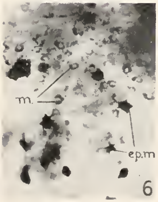

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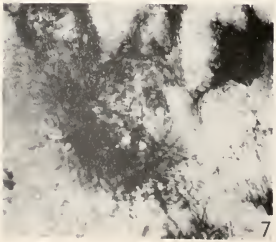

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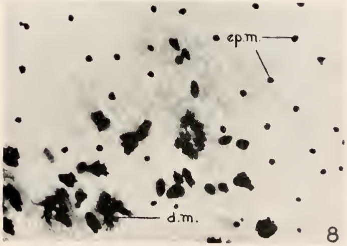

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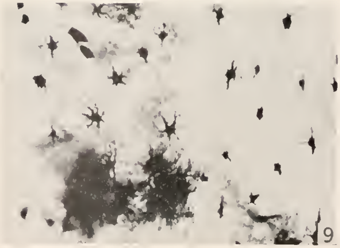

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PLATE I

If intermedin injections were carried out for a considerable length of time in an hypophysectomized animal, one would expect to obtain the same dark color possessed by a normal animal due to the gradual deposition of epidermal melanin.

Other Snakes

A procedure similar to that outlined above was carried out with the other species of snakes mentioned. In all these forms suitable scales can be found which have small epidermal melanophores superimposed on a light dermis. These cells, however, account for very little, if any, color changes, yet will respond in the same way as the rattlesnake to hypophysectomy and intermedin injection. The color pattern of these snakes is accounted for primarily by a great concentration of various dermal chromatophores which have not been studied.

Discussion

The primary concern of this study is to point out that the pituitary and more specifically the pars intermedia of the rattlesnake is responsible for the dispersed phase of both types of melanophores. Noble and Bradley (1933) were first to associate the paling response of the lizard, *Hemidactylus,* with the removal of the pituitary. Similar observations were later made in much greater detail on *Anolis* by Kleinholz (1935, 1938*a, b*), and on the horned toad, *Phrynosoma,* by Parker (1938). Little seems to be known concerning the normal color changes in snakes. The experimentally induced metachrosis described above is relatively slow but definite, and this group of reptiles may now be included in the ever increasing number of vertebrates which have a pituitary regulation of the melanophores.

The occurrence of epidermal melanophores and dermal melanophores whose processes reach into the epithelial layer has long been recognized, but has received little attention (Kerbert, 1877; Krauss, 1906; Fuchs, 1914; Schmidt, 1917; Lange, 1931). They seem to be rare in the lizards (Schmidt, 1917) and are probably obscured in most snakes by the dark, dermal chromatophore layer. In the rattlesnake, however, these melanophores are especially striking, since their grouping and distribution seem to be responsible for the whole color pattern superimposed upon a uniform yellow, dermal guanophore layer. The melanophores are not crowded and are easily seen, since all their branches extend into the epidermal stratum. This species is consequently peculiarly adapted for this study. The other snakes studied are on the whole rather dark-colored forms and the activity of the epidermal melanophores can only be recognized in a few light scales and even here they contribute little to the color pattern.

All authors who have studied the shed reptile skin, especially that of the snakes (Leydig, 1873; Werner, 1892; Lange, 1931) have observed the close coincidence between the melanin pattern of the shed skin and the pattern of the underlying layers. This can be followed especially well in the rattlesnake where the melanin deposits of the stratum corneum appear to coincide with the spread of each individual dermal and epidermal melanophore. Such a situation poses the still unanswered question of how this melanin is deposited. Is the melanin formed independently by the epidermal cells, as seems to be the case in various regions of the amphibian skin, or is it actually "injected" into the epidermal elements by the branches of the melanophores as Strong (1902) has described for the birds? In the snake the close association of all melanophore branches with pigmented epidermal cells suggests very strongly a mechanism of melanin deposition as in the bird feather. If this is actually the case, then one could expect complete cessation of all pigment deposits in an hypophysectomized animal, since the pigment is completely concentrated and would never reach the upper layers of the stratum germinativum where it seems to be normally laid down. Although all hypophysectomized animals remained permanently pale as long as four months after the operation, closer examination revealed slight melanin deposits in the epidermal cells. However, this does not necessarily invalidate this theory, since all these snakes received periodic intermedin injections and consequently might have had an opportunity to deposit pigment during these intervals of melanophore dispersion.

To what extent the color changes in the snakes can be compared with the relatively sudden changes described for the lizard group is difficult to state. In *Anolis* (von Geldern, 1921; Kleinholz, 1938*a*) the light (green) and the dark (brown) phases are due to the concentration and dispersion of pigment in the dermal melanophores. These melanophores never extend into the epidermis and achieve their effect by exposing or masking the green-reflecting chromatophores of the dermis. This metachrosis may be accomplished in a matter of minutes. In the rattlesnake the light and dark phases are accomplished experimentally in a similar manner, but the processes of the melanophores lie primarily in the epidermis, give rise themselves to definite patterns and are rather slow to react. As pointed out before, another factor must be considered in the light and dark phase of the snake. This is the heavy accumulation of melanin granules in the epidermal cells, which plays a minor or questionable rôle in *Anolis*. The melanin accumulation is especially noticeable in the darkening of snakes before they shed (Fig. 2), for at this time the future stratum corneum has already formed under the old layer and undoubtedly contributes to the darkened condition as the new layer already carries considerable pigment. The amount of melanin deposits, however, seems to be in some way correlated with the melanophore activity, since hypophysectomized animals lay down very slight amounts of pigment, or no pigment at all, and remain permanently pale.

Exact studies concerning the effect of temperature and light on the chromatophore activity in snakes have not been found in the literature. The rôle of these two factors in the melanophore regulation of various lizards has recently received much attention by Kleinholz (1938*a, b*), Parker (1938), and Atsatt (1939), but whether or not snake melanophores will respond in a similar manner awaits further study.

Summary

1. In the prairie rattlesnake, *Crotalus v. viridis* Raf., the background of the skin is a homogeneous yellow-white color reflected from the evenly distributed dermal guanophores. The dark pattern of this snake is formed by various distributions of melanophores and pigmented epidermal cells superimposed upon this background.

2. Two main types of melanophores are found in the skin. One is relatively small and resides primarily in the epidermis; the other is much larger, structurally different, and retains its cell body in the dermis, but sends its ramifying processes into the epidermal layer.

3. Both of these melanophores appear to be associated with the deposition of melanin granules in the keratinizing portion of the epidermis, since (*a*) the distribution of its melanin deposits coincides with the pattern of the underlying melanophores, and (*b*) the rate of pigment deposition is greatly reduced after hypophysectomy.

4. The removal of the pituitary or the pars intermedia alone causes a permanent paling due to the complete concentration of the melanophore pigment. This paling, however, is more evident after the shedding of the old keratinized epidermal layer carrying previously deposited melanin.

5. Intraperitoneal injections of intermedin from the chicken pituitary will produce complete melanin dispersion in the melanophores of an hypophysectomized animal.

6. Preliminary observations on four other species of snakes indicate a similar pituitary regulation of the epidermal melanophores.

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DIPLOID AND ANDROGENETIC HAPLOID HYBRIDIZATION BETWEEN TWO FORMS OF RANA PIPIENS, SCHREBER[1]

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Introduction

One of the better methods for examining nucleo-cytoplasmic relationships is to combine identical nuclei with different cytoplasms. This can be achieved in a number of ways. For example, if the gametes of two different species are brought together to form reciprocal diploid hybrids, it is expected that at least in the early stages such hybrids will have identical nuclei and different cytoplasms. This arises from the fact that in most cases the maternal parent contributes practically all of the cytoplasm. Differences which may appear in the development and heredity of the reciprocals can therefore be related to differences in the egg cytoplasms of the two parent forms. If such cytoplasmic differences are observable and measurable, the possibilities are obvious.

The same end is achieved by combining the male nucleus of one species, subspecies or race with the nucleus-free egg cytoplasm of the same species and another species, subspecies or race. If the androgenetic or merogonic[2] homospermic haploid and heterospermic haploid resulting from this procedure show dissimilarities, these must be related to cytoplasmic differences.

It is scarcely necessary to point out that the results of such procedures seldom if ever satisfy the preconceived possibilities. The results from reciprocal diploid hybrids may be limited by an incompatibility of combined nuclei or nuclei and cytoplasms; or, where this is not the case, by the absence of sufficient cytoplasmic difference to produce an effect. And heterospermic haploids are usually less satisfactory. In the best of circumstances haploid organisms develop poorly and can only be produced in a limited group of materials. Apparently as a result of

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[1] Data obtained, in part, from experiments performed during tenure of National Research Fellowship at Princeton University.

[2] The term androgenetic refers to the development of the *whole* egg with only the male nucleus functional; merogonic refers to the similar development of an egg-*fragment* (Wilson, 1925).

a high degree of incompatibility between nucleus and cytoplasm, the development of most androgenetic species hybrids is extremely abnormal and ceases in the earliest stages.

Despite such results, the possibility remains that hybridizations with heretofore untried material such as the North American Salientia may reveal one or more compatible combinations with the desired qualities. Experiments to test this possibility have been made and the following pages report one such investigation. Two distinct but closely related forms of the genus *Rana* have been combined reciprocally to form diploid and androgenetic haploid hybrids. The results form an interesting addition to the existing data on nucleo-cytoplasmic relationships.

The author is very grateful to Prof. G. Fankhauser for helpful suggestions and criticisms.

MATERIALS

The gametes for these hybridization experiments were derived from two distinct forms of frog, one collected from the meadows of northern Vermont, the other from the immediate vicinity of Philadelphia. Both forms are commonly referred to as *Rana pipiens* and possibly represent different races or subspecies of that species. More attention will be given to their probable relationship in the discussion.

That the two forms are distinct is indicated by their general characteristics (Figs. 1 and 2), and also by the results of these experiments. These same features also indicate that the two are closely related. Therefore, as a temporary assumption and to facilitate the description of the experiments, the frogs are being considered as northern and southern forms or races of the same species. As such they will be referred to in the succeeding pages of this report.

A brief description, supplemented by Figs. 1 and 2, will indicate their major differences and similarities.

The northern form (from northern Vermont) is generally larger and, relative to its body size and weight, it has shorter jumping legs than the southern form. The head is obtuse; the vocal sacs on the male are less apparent; the dorso-lateral folds are broad; the skin is thick; the palmation is full. Distinctive features of pigmentation include spots that are larger and surrounded by a green or yellow border; the cross-bars on the tibia are generally complete; the posterior border of the thigh is marked by black spots on a continuous white background; the tympanum does not show a central light spot with the same clarity as in the southern form.

The southern form (from the vicinity of Philadelphia) is generally smaller and, relative to its body size and weight, its legs are longer. The head is more accumate; the vocal sacs are thin-walled and usually

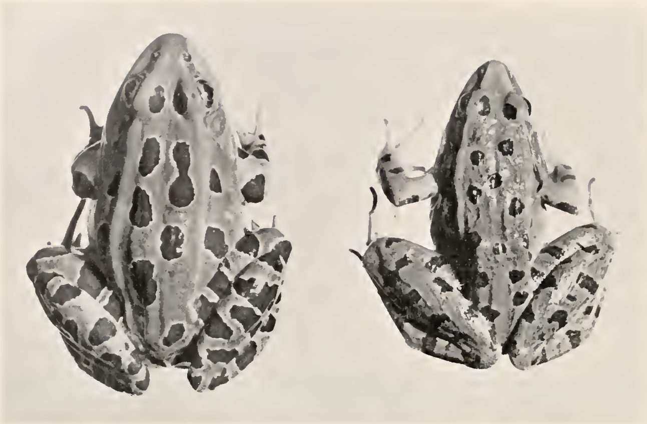

FIG. 1. Photographs of representatives of northern (left) and southern (right) forms of *R. pipiens* used in these experiments.

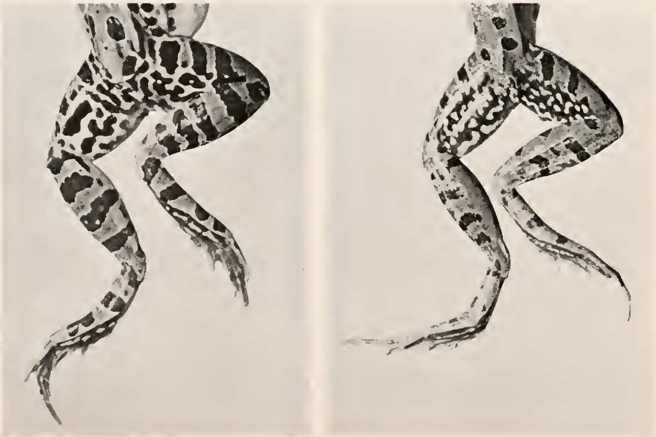

FIG. 2. Photographs showing pigmentation of jumping legs, northern (left) and southern (right).

apparent; the dorso-lateral folds are narrow; the skin is thin; the palmation is shallow. Distinctive features of pigmentation include spots that are smaller and, under the same laboratory conditions as the northern form, not surrounded by clear borders; the cross-bar markings on the tibia are generally interrupted along the dorso-lateral surface; the posterior surface of the thigh is marked by white spots on a continuous black background; and the tympanum generally shows a central light spot.

METHODS

The eggs were obtained in every case from frogs which had been induced to ovulate by frog pituitary injections. As much care as possible was taken to avoid removing the eggs in the immature or over-ripe condition. The sperm for insemination were obtained by macerating the testes in 10 per cent Ringer's solution and every precaution was taken against contamination of one suspension with sperm from another.

The eggs of each frog were inseminated in two batches, the first with sperm of the same form, the second with sperm of the other form. Thus, in all, four batches of eggs were inseminated. An interval of 15 to 20 minutes was allowed to elapse between each insemination to provide time for removing the egg pronucleus from a number of eggs of each batch. By this procedure 8 different types of embryos were produced. These are listed below with the designation used for each in the balance of this report.

Homospermic diploids of the northern form	n
Homospermic haploids of the northern form	$n/2$
Heterospermic (hybrid) diploids from eggs of northern form and sperm of southern form	ns
Heterospermic (hybrid) haploids from cytoplasm of northern form and nucleus of southern form	$(n)s/2$
Homospermic diploids of the southern form	s
Homospermic haploids of the southern form	$s/2$
Heterospermic (hybrid) diploids from eggs of southern form and sperm of northern form	sn
Heterospermic (hybrid) haploids from cytoplasm of southern form and nucleus of northern form	$(s)n/2$

The egg pronucleus was removed with a fine glass needle as described in a previous report (Porter, 1939). Adequate numbers of pure and hybrid haploids were thus easily prepared (Table I).

All embryos were kept under identical conditions of temperature (19.4° C.) and space. In fixation of representative forms for a permanent record, a mercuric chloride, acetic acid, and formaldehyde mixture was generally used. The same sequence and time intervals were observed in fixation as had been observed in fertilization. Thus it was assured that all animals fixed at the end of a period of time were of the same age.

RESULTS

The description which follows is based upon observations made in the experiments listed in Table I. The possibility that the same results could occur by coincidence in all four series of crosses is slight if not negligible. The analysis is confined to such characteristics as were apparent from external examination and only those characteristics which were uniformly shown by the animals in all four groups are stressed in the succeeding paragraphs.

TABLE I[3]

Exp.	Date	Number of homospermic haploids produced	Number of heterospermic (hybrid) haploids produced	Treatment
1.	Jan. 9, 1939	23 $n/2$ 21 $s/2$	41 $(n)s/2$ 35 $(s)n/2$	Preliminary comparison of living animals made throughout development. Representative embryos fixed at end of 3, 5, 7, 9, 10 and 11 days.
2.	Jan. 17, 1939	29 $n/2$ 37 $s/2$	30 $(n)s/2$ 44 $(s)n/2$	Living animals compared throughout development. Representative forms fixed at end of 2, 3, 4, 5, 6, 7 and 8 days. Diploid hybrids and controls carried through metamorphosis for examination of inheritance.
3a.	Feb. 15, 1939	37 $n/2$ 24 $s/2$	48 $(n)s/2$ 43 $(s)n/2$	Living animals compared throughout development. Special attention given to gastrulation and neural tube formation. Representative forms fixed at end of 36, 43, 48, 51, 53, 55, 57, 59, and 61 hours and at 3 and 4 days.
3b.	Feb. 15, 1939	21 $n/2$ 45 $s/2$	36 $(n)s/2$ 41 $(s)n/2$	Living animals compared. Special attention given to study of older stages. Material fixed at end of 32, 54, and 60 hours and 3, 5, 6, 8, 9, and 10 days.

For greater clarity the description of the 3-day-old embryos is presented first. With the differences of these in mind the descriptions of the younger and older stages have greater meaning.

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[3] The same females were used as a source of eggs for experiments *3a* and *3b*. Otherwise, different parents were used in each cross.

Three-day-old Embryos

The following account is illustrated by the outline drawings in Fig. 3 and to them reference is constantly made.

The homospermic (control) diploids of the two races develop at approximately the same rate at 19.4° C. and, stage for stage, are comparable at the end of 72 hours. The differences, though real, are very slight and were clearly recognized only after repeated examination of material available. The northern diploids compared with the southern diploids show larger gill plates, a larger sense plate and larger mucous glands. Relative to body size the head of *n* is the larger. The neural tube is broader and stands up more distinctly in *n*. The tail-bud in *n* is smaller and directed more dorsally than in *s*, thus creating a deeper depression in the back of *n*. In relation to head size, the abdomen of *s* is larger than that of *n*. To these differences it can be added that the head flexure dorsal to the posterior margin of the gill plate is more pronounced in *s* than in *n*.

The homospermic (control) haploids of the two races, as is normal for haploids, are retarded in their development. Compared with each other they show in an exaggerated form the same differences that were given for the diploid controls.

The heterospermic (hybrid) diploids show approximately the same rate of development as the homospermic diploids and as each other. They differ in body proportions and show in accentuation the differences which are difficult to see between the pure diploids of the two forms. A greater proportion of *ns* consists of head structures than in the reciprocal hybrid. Conversely, a greater proportion of *sn* consists of abdomen and tail-bud. The mucous glands and sense plate are larger in *ns* and, posterior to the medulla, *ns* shows a smaller neural tube which terminates in a smaller and more dorsally directed tail-bud.[4]

The heterospermic (hybrid) haploids show in most exaggerated form the differences which have been referred to as existing between control diploids and haploids and more distinctly between the hybrid diploids. It is readily apparent that oral suckers, gill plates, and sense plate are greatly enlarged in $(n)s/2$. Relative to head size, the abdomen and tail-bud of $(s)n/2$ are much larger than the same structures of $(n)s/2$. It can be further noted that the head of $(s)n/2$ is flexed more ventrally than $(n)s/2$ and the back of the latter is convex while in the former it

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[4] If these experiments had been confined to the production and study of diploid hybrids, it is doubtful if the differences would have been considered great enough to warrant any conclusions. Supported by the evidence from androgenetic hybrids, however, the significance of the differences is unquestionable.

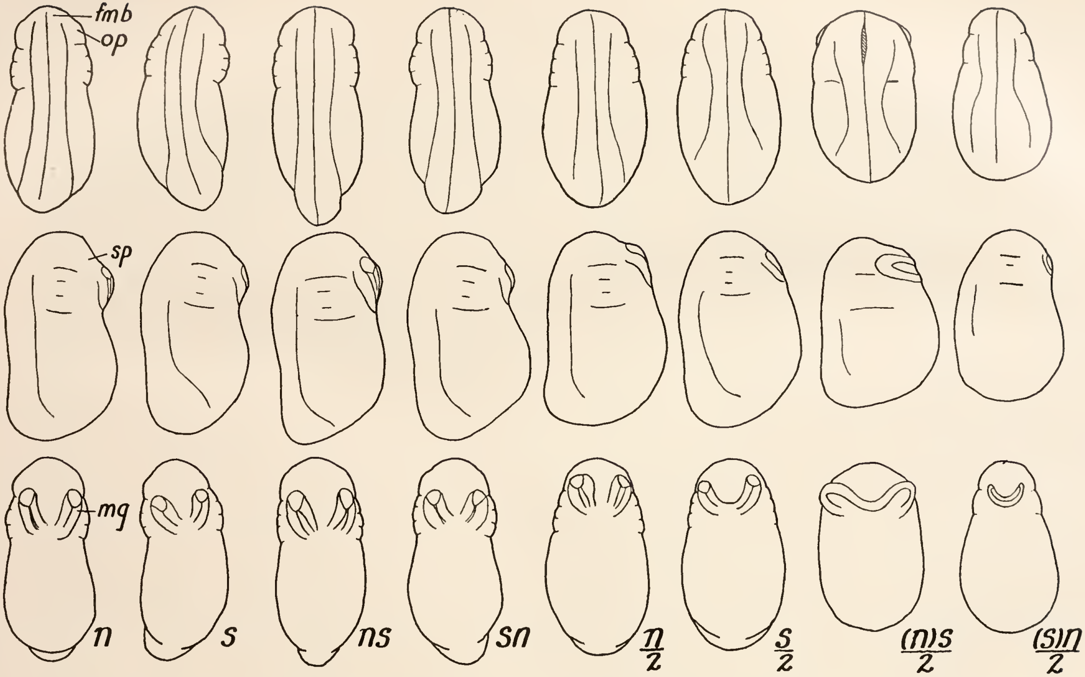

FIG. 3. Outline camera lucida drawings of dorsal, lateral and ventral views of 72-hour-old embryos. From left to right the drawings represent: *n*, homospermic diploid control of northern form; *s*, homospermic diploid control of southern form; *ns*, heterospermic diploid from northern egg and southern sperm; *sn*, heterospermic diploid from southern egg and northern sperm; *n/2*, homospermic haploid control of northern form; *s/2*, homospermic haploid control of southern form; (*n*)*s/2*, heterospermic haploid (androgenetic hybrid) from northern cytoplasm and southern sperm; (*s*)*n/2*, heterospermic haploid from southern cytoplasm and northern sperm. Magnification, 12.5 ×. The same sequence and magnification are observed in Figs. 4, 5, 6, and 7.

It can be seen that head structures (fore- and mid-brain regions, *fmb*, optic vesicle outpushings, *op*, sense plate, *sp*, and mucous gland, *mg*) are larger in *n*, *ns*, *n/2*, and (*n*)*s/2*, than *in s*, *sn*, *s/2*, and (*s*)*n/2* respectively. The tail-bud, *t*, tends to be larger in the latter. These differences are clear between the reciprocal heterospermic diploids, *ns* and *sn*, and pronounced between the is concave. These differences are the expression of the decidedly dissimilar embryology of the two reciprocal heterospermic haploids and not a difference in age or stage.

Summary.—In general the combinations which include cytoplasm of the northern form are characterized by larger head primordia and smaller posterior axial structures than are observed in those with southern cytoplasm. Such differences, only slightly apparent in the pure diploid controls, become progressively more accentuated in the homospermic haploids, the heterospermic diploids and in the heterospermic haploids.

It is of interest to observe now the earlier and later expressions of these general differences as shown by an examination of the earlier and later stages in the ontogeny of the various combinations.

Neural Tube Formation

The description under this heading is derived from a comparative study of living material and of representative embryos of the eight different types fixed at intervals of two hours from 51 to 61 hours after insemination. Illustration is provided by outline figures 4, 5, and 6 which are respectively representative of developmental stages reached at the end of 55, 59 and 61 hours.

The homospermic diploids, during this period, are very similar both in character and rate of development. As the neural plate is outlined, it becomes apparent that its anterior portion plus the sense plate are larger in *n* than in *s*. These differences increase in clarity as the neural folds are elevated and gill plates appear (Figs. 5 and 6). At this latter stage, *s* flattens dorsally and shows a greater elongation of that portion of the neural groove posterior to the gill plates. At the same time the neural plate and folds are more distinctly elevated in *s*. Although the neural plate and folds may be outlined in *s* slightly in advance of *n,* the closure of the folds is more rapid in the latter. During neurulation the blastopore of *s* is bounded laterally by distinctly thickened lips.

The homospermic haploids show in exaggerated form the slight differences existing between the diploid controls. In equivalent stages (55 hours) the neural folds of *n/2* are thicker, the sense plate and other primordial head structures are larger than in *s/2,* whereas the latter shows a greater elongation of the neural plate, especially that portion of it determined to be spinal cord. In *s/2* the neural folds show a greater elevation, the dorsal surface straightens or flattens out, and pronounced lateral lips bound the blastopore. Besides stage-for-stage structural differences, the differences in rates of separate morphogenetic processes are also accentuated. For example, it is noted that *s/2* completes gas-

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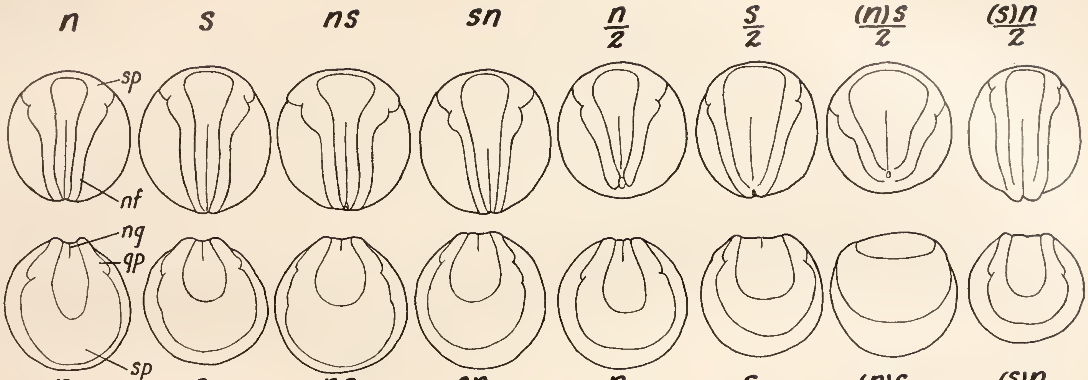
n
s
ns
sn
n/2
s/2
(n)s/2
(s)n/2
sp
nf
ng
gp
sp

5

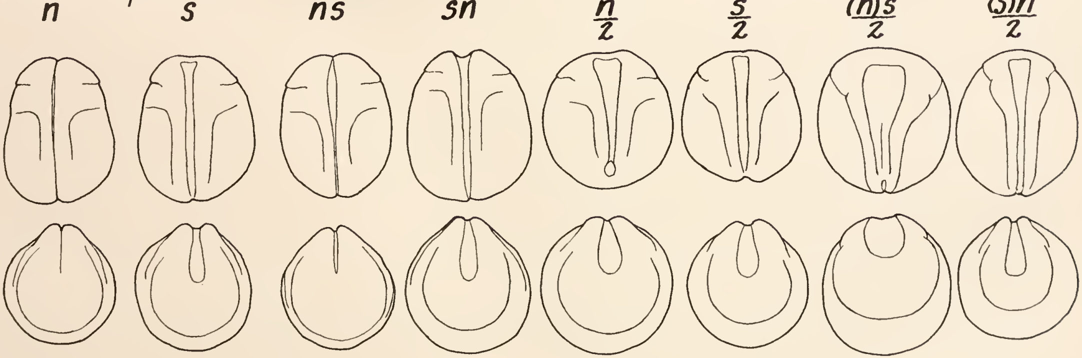
n
s
ns
sn
n/2
s/2
(n)s/2
(s)n/2

FIG. 4. Dorsal and anterior views of 55-hour-old embryos. It can be noted that, relative to size of abdomen, the head primordia (sense plate, *sp.*, gill plate, *gp.*) of *n*, *ns*, *n*/2, and (*n*)*s*/2 are larger than the same of *s*, *sn*, *s*/2 and (*s*)*n*/2. Neural folds, *nf*. Neural groove, *ng*.

FIG. 5. Dorsal and anterior views of 59-hour-old embryos.

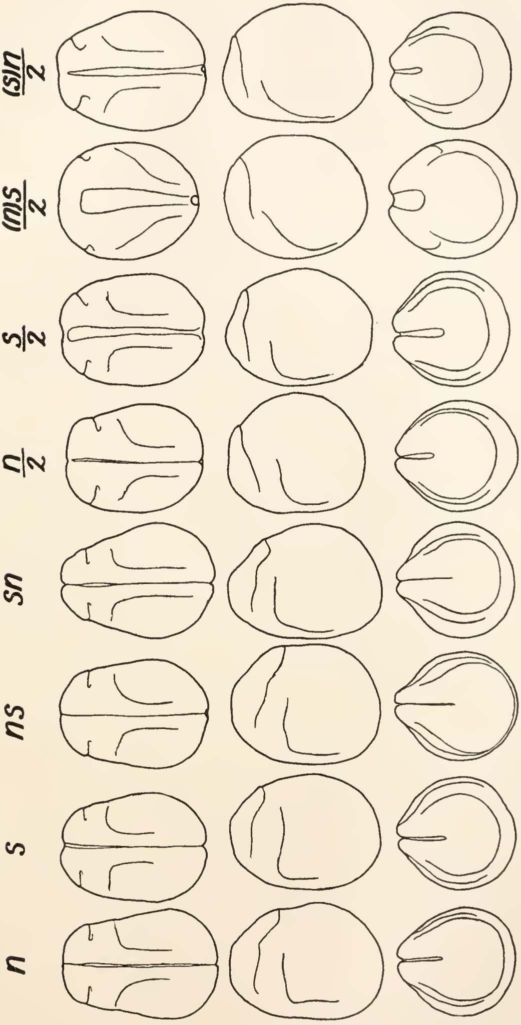
n
s
ns
sn
n/2
s/2
(n)s/2
(s)n/2

FIG. 6. Dorsal and anterior views of 61-hour-old embryos.

trulation considerably ahead of *n/2* and only slightly after *s*, whereas in *n/2* the neural folds appear to close slightly before they do in *s/2*.

The heterospermic diploids show clear-cut differences. Compared with each other in the early stages of neurulation, it is apparent that the *ns* forms have a shorter neural plate which is abnormally broad at the anterior end. The reciprocal *sn*, on the other hand, has a long and narrow neural plate. As the two differ from each other so do they differ from their maternal control diploids though to a lesser degree. Other features of dissimilarity include neural folds which are larger in *ns* than in *sn* and which are more distinctly elevated in *ns* than in the control diploid, *n*. In this latter respect they approximate the condition noted above as apparent in the paternal diploid control. As neural tube formation continues, the greater size of the head primordia and the shorter neural plate and groove are maintained in *ns*. Though the neural plate and folds are outlined almost simultaneously in these reciprocal hybrids, the folds come together in *ns* slightly before they do in *sn*. The lateral borders of the blastopore are swollen in *sn* to form lips as in *s* and *s/2*.

The heterospermic haploids show very striking differences during the development of the neural tube. At the end of 51 hours (not illustrated) (*s*)*n/2* has a dorsal flattened surface, abnormally straight from anterior to posterior ends. The neural plate is clearly outlined and is very narrow. Gastrulation has been completed, and there are extremely pronounced lips on both sides of the blastopore. Contrasting with this, the reciprocal (*n*)*s/2* is considerably retarded. The yolk plug is still apparent and the limits of the neural plate are not visible. The (*n*)*s/2* embryos are flattened dorso-ventrally and present a large, swollen appearance. By the end of 55 hours the neural plate of (*n*)*s/2* has been outlined. It is as broad as it is long and that portion designated to become neural tube is extremely short. The yolk plug persists. At this same time in (*s*)*n/2* the neural plate has lengthened and the neural folds have approximated to some extent. At 59 hours (*n*)*s/2* continues to show a short, broad neural plate, bounded by prominently elevated neural folds. This latter feature is a characteristic of *s* embryos and its appearance in these (*n*)*s/2* embryos represents the appearance of a specific paternal character. In the reciprocal it is not shown. It is a feature which will be easier of description and analysis when sectioned material is available. By 61 hours the neural folds of (*n*)*s/2* have started to approach and subsequent observations have shown that once started this process proceeds more rapidly here than in (*s*)*n/2*. At this time and later there is little elongation of the neural plate in (*n*)*s/2* and the yolk plug still persists in some cases. The sense plate and gill plates are abnormally large. The structure of the reciprocal hybrid (*s*)*n/2* at 61 hours is characterized by neural folds about ready to close, an elongate neural tube, and extremely small head primordia which foreshadow the diminutive head size of later stages.

Summary.—In summarizing, a few generalizations can be made. Those combinations which include cytoplasm of the northern race, including the diploid controls, are characterized by: (*a*) neural plates which when outlined tend to be shorter, and broader anteriorly, and (*b*) head primordia which are larger. The reciprocal combinations with cytoplasm of the southern race are, on the other hand, characterized by: (*a*) longer and narrower neural plates, (*b*) smaller head primordia, and (*c*) pronounced lateral lips on the blastopore. These differences become increasingly apparent as one compares respectively the diploid controls, the haploid controls, the reciprocal hybrid diploids, and the reciprocal androgenetic hybrids.

At one stage in the development of the neural folds it is apparent that they are more sharply delimited and distinctly elevated in the diploid of the southern form. This characteristic is repeated in the hybrid diploids and in the androgenetic hybrids containing the southern nucleus. It seems to represent, therefore, an inheritable embryonic characteristic capable of expressing itself in the foreign cytoplasm of the northern race. More careful analysis of this phenomenon is needed.

There are also to be noted slight differences in the times of occurrence and rates of the same morphogenetic processes. Relative to blastopore closure the neural plate is outlined earlier in those combinations with northern cytoplasm. Relative to time after fertilization, however, this may be later. Once clearly outlined the neural folds of the combinations with the northern cytoplasm seem to close more rapidly.

Gastrulation

This phase of the embryology of these various combinations was studied from living material and from representative forms fixed at the end of 36, 43, and 48 hours. A few differences between the gastrulae of those forms with northern cytoplasm and those with southern cytoplasm occur consistently (excepting the diploid controls where they are not sufficiently pronounced to be clearly evident) and become progressively more pronounced in haploid controls, heterospermic diploids, and heterospermic haploids. Those combinations with the cytoplasm of the southern race show a larger gastrular angle, a smaller completed blastopore, epiboly largely from the dorsal and lateral borders of the blastopore, and toward the end of gastrulation, an increasing thickening of the lateral blastopore lips. Those combinations with northern cytoplasm show a smaller gastrular angle, a larger blastopore, epiboly from all sides of

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the blastopore, and thin blastopore lips. Gastrulation appears to begin earlier in *s, s/2, (n)s/2,* and simultaneously in *sn* and *ns*. Observations recorded on this feature and on the rate of gastrulation are not sufficiently extensive to be conclusive.

It would seem that the greater gastrular angle and the greater epiboly of the dorsal lip in those haploid and hybrid embryos with the southern cytoplasm are the early abnormalities related to the longer neural plate of later stages. It also appears that the thickened lateral blastopore lips of these same forms are the early expression of the larger tail-bud and somites of later stages (Bijtel, 1931). The opposites of these same features in those forms with the northern cytoplasm are probably related to the shorter neural plate and smaller tail-buds of their later stages.

Older Stages (4–10 Days)

The studies reported in this paper have been largely devoted to the younger stages hence only the most general features of the older stages will be described under this heading. Reference should be made to Fig. 7.

The homospermic diploids of 4 and 5 days continue to show the slight differences which existed between the 3-day-old embryos. In the older stages, however, these differences become increasingly subtle. Relative to body proportions, the head of *n* remains larger while the tail of *s* is more elongate and larger in relation to the rest of the embryo. The dorsal concavity of *n* persists in greater prominence than in *s*.

The homospermic haploids differ in the older stages, as they had in the earlier stages, in relative size of body parts. The differences are similar to but more distinct than those occurring between the diploid controls.

The heterospermic diploids demonstrate more clearly the perpetuation of early differences. The combination, *ns,* persists in showing at various ages a larger head with larger mucous glands and a smaller dorsally directed tail. The converse of these features are shown by the reciprocal. Such differences are retained into the later stages of development, especially the relative head and tail size. Clear-cut appearance of paternal characteristics is recognized first in stages showing chromatophore patterns.

The heterospermic haploids, as in the younger stages, show the most striking differences. It is recognized, however, that these differences are less pronounced in the older stages suggesting some regulation. It is easily noted that the head of *(n)s/2* and its component structures remains larger and the tail remains smaller and directed dorsally. The androgenetic hybrid, *(s)n/2,* on the other hand, is characterized by a

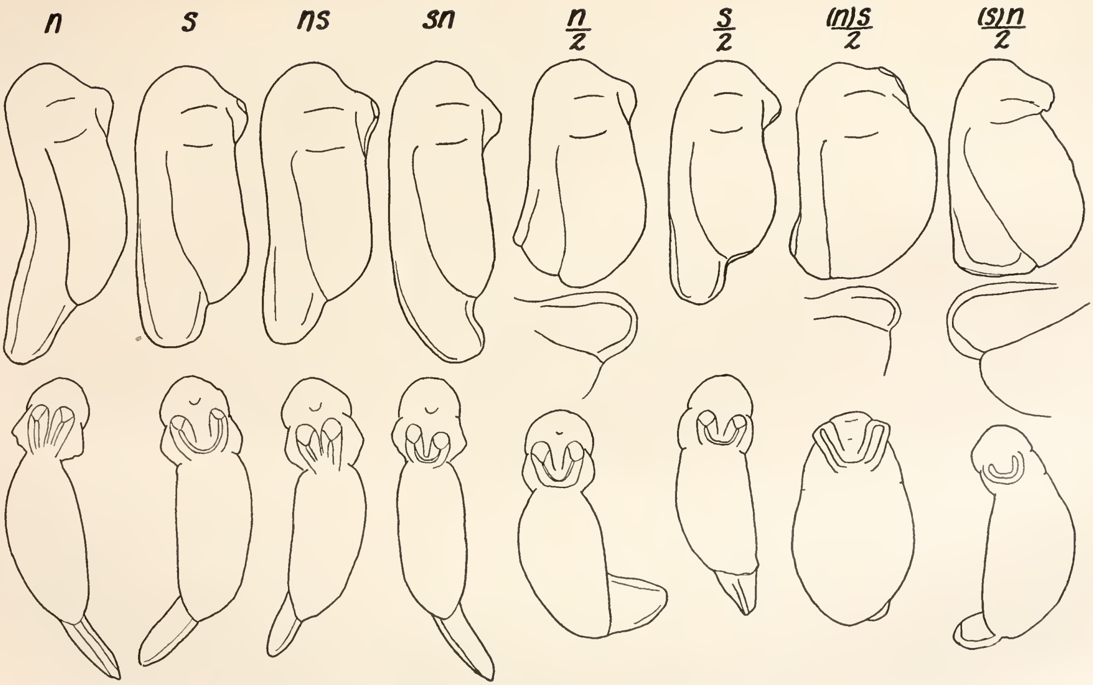

Fig. 7. Lateral and ventral views of 4-day-old embryos. It can be noted that early differences in relative body proportions persist in the various combinations.

small head and a ventrally directed tail which is referred to as larger because of the broad heavy somite mass at its base. These features were foreshadowed in the early embryonic development.

Summary.—Those combinations with cytoplasm of the southern race tend to have smaller head structures and, relative to body size, larger tails than the reciprocals with northern cytoplasm. Such features are doubtless the expression of earlier embryonic differences in the size of head primordia and tail-buds.

Survival of Various Combinations

Since representative embryos were sacrificed for fixation at various intervals, no definite data can be given to demonstrate survival value. Nevertheless, the observations made permit the following statements.

The heterospermic hybrids, in the majority of cases, develop up to and through metamorphosis. Beyond that stage no data are available.

The homospermic haploids of the two races demonstrate approximately the same viability. They continue their development, on the average, for from 8 to 12 days up to approximately stage 24 (Shumway, 1940).

The heterospermic haploids develop through the early stages showing only a small percentage of deaths. About one-fourth to one-third fail to hatch and of those which hatch the majority live for from 7 to 11 days or up to stages 22 and 23 (Shumway, 1940). They are slightly less viable than the haploid controls. In a small percentage of cases a gill circulation is established and in scattered cases growth continues sufficiently long to show the first guanophores. No differences in viability were recorded as existing between the two reciprocal combinations.

Identification of Haploids

The haploids were identified as such solely on the basis of the type of development. In an earlier study (Porter, 1939) it was shown that embryos which arise from operated eggs can be expected to develop as haploids in 90 per cent of the cases. Furthermore, such haploids were found to show certain definite characteristics when compared with their diploid controls. Hence, in these experiments, it has not been considered necessary to make a complete cytological examination of every embryo which developed from an operated egg, especially since group characteristics rather than individual characteristics have been considered. The isolated cases of diploidy which did appear among the embryos from operated eggs were readily identified by their development, cell size, etc.

In order to establish the chromosome count of the southern form, the tail-tips of several haploids were examined. The examinations made indicated 13 to be the haploid count. This is the same as for the northern form determined in an earlier study.

Other Observations

In the case of two of the above-described experiments, surplus diploid embryos both pure and hybrid were kept for examination as older tadpoles and as metamorphosed frogs. The pure diploids showed differences characteristic of the northern and southern forms; the hybrid diploids showed blended inheritance with indications of stronger paternal influence in certain features of pigmentation. Thus despite the blending the reciprocal hybrids were distinguishable. These observations indicate that at least the differences in pigmentation between the two parent forms are related to differences in nuclear factors.

Two further observations, which, because of the small amount of evidence supporting them, must be considered as very preliminary, are briefly described. They are presented because of their interest as possible leads for experiments aimed at determining the nature of the factors responsible for the peculiar development of the hybrid embryos described above.

Since the frogs used were obtained from widely separated northern and southern points, it was considered of interest to examine the effect of high temperatures. Embryos representative of the 8 different combinations treated above were placed in a warming oven at 28°–29° C. Those combinations with cytoplasm of the southern form were not apparently damaged and developed in the typical manner, whereas those combinations with northern cytoplasm were markedly affected and only a very small percentage of the original number developed through 6 days. Controls kept at 19.4° C. developed normally.

Cytological examination of a few pure diploid 3-day-old embryos revealed some interesting differences in nuclear size and size of yolk granules. Measurements of nuclei of identical tissues of the two forms showed those of the northern to be the smaller. Measurements of the yolk granules revealed those of the northern to be much the larger.

DISCUSSION

The discussion which follows will be confined to a consideration of the probable relationship of the animals used and to the more general aspects of the cytoplasmic and nuclear influences demonstrated. A detailed and inclusive treatment must await the accumulation of data from a more thorough study of these and similar hybrids. In a sense, then, this constitutes a preliminary report.

When the experiments were first undertaken the parents were thought to represent two distinct species. This conclusion was based on differences which the animals showed and also on the authority of amphibian taxonomists (Kauffeld, 1937; Stejneger and Barbour, 1939). An examination of the literature soon revealed, however, that considerable confusion exists in the classification of the leopard frogs or frogs of this type resident in the eastern states and possibly over a wider area. Differences between those forms resident in northeastern and those in the southeastern states have long been recognized, but it appears that sufficient material from a variety of localities has never been examined to make a conclusive analysis of the species. A brief reference to the writings of a few authorities on Salientia classification will serve to illustrate this confusion.

It should be recalled that the southern forms used in these experiments were collected in the vicinity of Philadelphia and the northern forms in northern Vermont. From its place of collection, the southern form doubtless coincides in appearance with that type early described by Schreber (1782) as *Rana pipiens* (Kauffeld 1936 and 1937). Later, Cope (1889), from examination of forms collected in a variety of localities, chose to describe the leopard frogs under three subspecies. The southernmost type he called *Rana virescens sphenocephala;* the type from the Atlantic coast *Rana virescens virescens* (probably same as Schreber's *R. pipiens* and the Philadelphia type of this study); and the type of northern distribution he called *Rana virescens brachycephala* (his description of which coincides perfectly with the northern form used in these experiments). More recent authors (Wright, 1933; Dickerson, 1906) have pictured and described the northern form as the typical *R. pipiens* and both it and the southern form have been considered as such by teachers and investigators alike. Most recently Cope's nomenclature has in part been revived, only instead of using a subspecies classification, the three types have been placed in separate species. Thus the most southern form is called *R. sphenocephala,* the Philadelphia form falls within the range of *R. pipiens* and the northern becomes *R. brachycephala* (Kauffeld, *loc. cit.,* and Stejneger and Barbour, *loc. cit.*).[5] It was on the basis of this latter classification that the frogs were originally considered to represent two species, *R. pipiens* and *R. brachycephala.* It is clear, however, that this classification is uncertain and consideration of some further points increases this uncertainty.

In the first place, it would seem that the two forms hybridize too successfully to be representatives of two distinct species. It is true that a few distinct species of the Salientia have been successfully hybridized (Born, 1883; Pflüger and Smith, 1883; Heron-Royer, 1891; Montalenti, 1933; Durken, 1938 and Moore, 1940) so that the successful crossing of these two forms, even if they represent distinct species, is not without parallel. What is unique is the result of androgenetic hybridization, for no case involving the Salientia has been reported in which the development of an androgenetic or merogonic species hybrid continued to the advanced stages obtained in these experiments.[6] In other words, the compatibility of the two forms is greater than would be expected of two distinct species.

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[5] In footnote, Stejneger and Barbour (1939) indicate that the whole sphenocephala-pipiens-brachycephala complex needs further examination and possible revision.

In the second place, it can be said that the characteristics of the two forms do not differ sufficiently to place them in separate species. Aside from body proportions, which is dealt with below, the major difference is one of pigmentation. This difference, it can be noted, does not involve the pattern but chiefly the size of the markings and intensity of the coloration. These are features which in other animals may vary considerably among races.

Finally, recalling that the two forms were collected from different northerly and southerly climates, and considering the points about to be discussed, the differences in body proportions likewise do not support a species relationship. Taxonomists have long been acquainted with certain generalizations known as the Bergmann and Allen rules pertaining to differences in size and body proportions which can be recognized between the northern and southern races of warm-blooded species. The former of these states that northern races are larger; the latter, that the southern races have relatively longer body projections. Within recent years an increasing volume of research examining racial and subspecies differences has shown that characteristics other than body size and proportions may likewise vary in an orderly and predictable manner with a variety of environmental gradients. Inclusive surveys of these phenomena are to be found in the recent writings of Goldschmidt (1940), Rensch (1936) and others. But among the species of animals examined for chains of racial differences or " clines " (Huxley, 1938), it appears that species of Amphibia have been regrettably absent. Schmidt (1938) reviewed some measurements of species of Salientia and noted that relative to body size the leg length was greater for those representatives of a species which were collected from the more southern localities. The small number of animals examined and the preserved condition of these did not, however, permit any definite conclusions. Measurements of unselected groups of the two forms used in these crosses show the same tendency of the northern form to have a heavier and larger body structure relative to leg length.

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[6] Baltzer (1920 and 1933) reports that from the combination of *Triton taeniatus* cytoplasm and *Triton palmatus* nucleus heterospermic haploids develop to stages showing good eye formation, pigment, small branching gills, and pulsating heart. Though this represents advanced development as compared with the usual result with different Salientia *species,* the stage reached does not seem to be the equivalent of that reached by the best of the heterospermic haploids obtained with these two *forms* of Rana pipiens.

It is possible that a thorough examination of the literature would reveal additional references to racial differences between frogs. For example, such differences are briefly mentioned in a paper by Pflüger and Smith (1883). Comparing the English race of *R. fusca* with the Königsburg race of the same species, they write:

> "Der englische braune Grasfrosch ist etwas kleiner und schlanker als der deutsche, weniger stumpfschnauzig und von zarterer Haut."

The similarity between these differences and those noted between the Vermont and southeastern Pennsylvania forms of *R. pipiens* is obvious. This similarity takes on added interest when it is noted that roughly the same climatic differences (as indicated by mean annual temperatures) exist between East Prussia (44° F.) and England (50° F.) as between Vermont (43° F.) and southeastern Pennsylvania (52° F.).

In view of these observations and the fact that racial variations accompanying climatic gradients have been found in a great many species of both the animal and plant kingdoms, it seems probable that species of frog when thoroughly examined will likewise show various clines with regard to temperature and other environmental factors. In the meantime it can only be maintained that the two forms used in these crosses probably represent two races of the same species.

If such is the case, the results of these experiments are of interest in demonstrating that racial differences involving body proportions can be recognized in early embryonic stages, and that at least some of the factors responsible for these differences exist in the cytoplasmic organization of the egg (see below). This observation and others which will probably be made from a more extensive examination of these and similar crosses may prove of interest to students who concern themselves with factors involved in species formation.

Experiments examining the relative rôles of the nucleus and cytoplasm in heredity have generally shown the nucleus to be the sole bearer of factors controlling the appearance of specific adult and juvenile characteristics. Some of these experiments have combined the nucleus of one species with the cytoplasm of another to form merogonic hybrids, attempting thus to demonstrate the presence of hereditary units in the cytoplasm. Among these, the studies of the Hertwigs, Boveri, Baltzer, Hadorn, and Hörstadius are well known and frequently reviewed. With the possible exception of Hadorn's (1936) results, the demonstration of cytoplasmic inheritance has not been conclusive. The development of the merogonic hybrids generally ceases very early and even where it continues to a stage showing distinct species characteristics, as in certain sea-urchin merogons, the intermediate condition of the characteristic can be considered as an abnormality resulting from a degree of incompatibility between the nucleus and cytoplasm (Hörstadius, 1936). The early cessation of development which characterizes amphibian merogonic hybrids is probably also the result of a severe incompatibility.

It would appear that by using more closely related forms than those belonging to different species this problem of incompatibility could be overcome. To some extent this is probably true, but in using members of different races or subspecies, it is necessary to sacrifice the clear-cut distinctions which usually exist between the embryonic stages of different species and which are not to be expected between different races. Hence, the problem is fraught with difficulties and it is doubtful whether material such as used in these experiments, though it should be thoroughly examined, will supply any evidence in support of cytoplasmic-borne units of heredity even if present.

As distinct from heredity, cytoplasmic influence on development has been and can be demonstrated. This influence has been considered as the effect of plasmatic organization and composition upon the expression of nuclear factors. To this category of cytoplasmic activity the results of these crosses probably belong. Experimental embryologists have long recognized a high degree of cytoplasmic differentiation in a variety of eggs and the maintenance of such differentiation undisturbed is known to be essential in many cases for normal embryonic development. The cytoplasm of the egg by its organization, therefore, exerts an influence on the appearance of the adult in so far as this appearance is determined by the characteristics of the early developmental stages.[7] Needless to say, the nature of this early cytoplasmic influence is not understood but every new demonstration of its presence offers new possibilities for its examination.

The consideration of the results of these experiments is facilitated if the development of the two control diploids is visualized as paralleling on opposite sides an average or mean type (Fig. 8). If the factors responsible for this slight departure from the mean are nuclear and the

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[7] In respect to even this cytoplasmic influence, it is to be remembered that considerable differentiation of the egg takes place in the presence of the maternal nucleus.

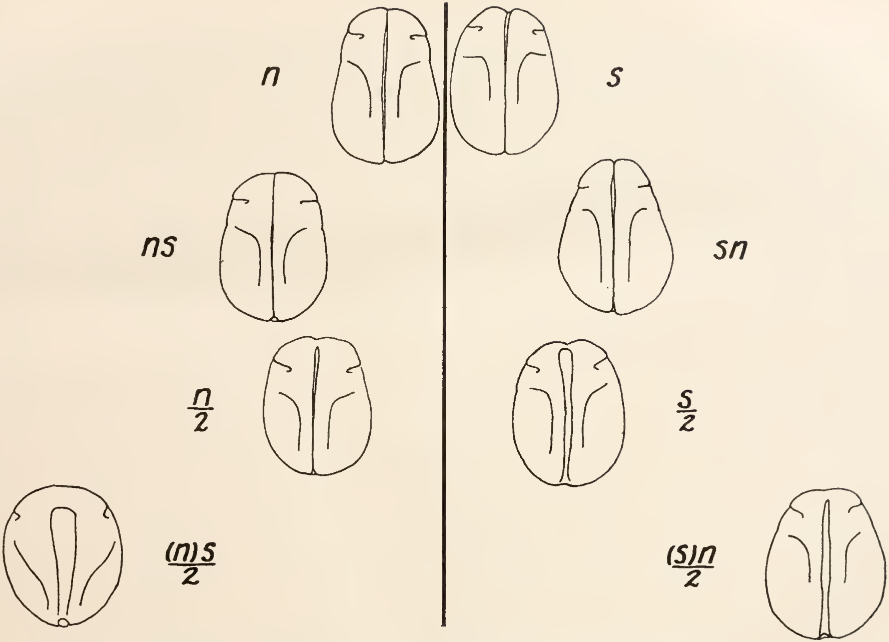

FIG. 8. Dorsal views of 61-hour-old embryos representing the development of the various nuclear-cytoplasmic combinations in relation to a hypothetical mean type, the central line. Those combinations with northern cytoplasm are on the left of the line; those with southern cytoplasm are on the right. The distance from the line represents the approximate degree of difference between the development of any one combination and the mean.

cytoplasms are perfectly neutral to nuclear control, then the diploid reciprocal hybrids would be expected to be identical and would in their development occupy a position coinciding with the hypothetical mean. Under the same conditions of nuclear control, the heterospermic haploid with the southern cytoplasm and northern nucleus would be expected to show the same development as the homospermic haploid of the northern form. Neither of these results is obtained. Instead, it is noted in the case of the reciprocal diploid hybrids that their development places them on opposite sides of the mean and at points more distant from the mean than their diploid controls. And in the case of the heterospermic haploids, the hybrid with the northern nucleus is not only further from the mean than the homospermic haploid of the northern form, but it is on the opposite side. Since the diploid hybrids can be considered as having identical nuclei and differing only in their cytoplasms, and since the same difference holds between the homo- and heterospermic haploids with nuclei of the same form, it follows that cytoplasmic influence is responsible for the dissimilarities existing between them.[8] Therefore, the eggs of the northern and southern forms differ in some property or properties of their cytoplasms.

Are the nuclei identical or do they also differ? If the nuclei are considered as being identical and responding solely to cytoplasmic influence, then the development of the reciprocal diploid hybrids should parallel the mean at the same distance as their respective diploid controls. Or, under the same assumed conditions of identical nuclei, the heterospermic haploid with the southern cytoplasm should be identical in appearance with the homospermic southern haploid. Again, the results indicate that the assumed condition of identical nuclei cannot be valid. On the other hand, the intermediate position of the diploid control between the mean and the hybrid diploid with the same cytoplasm indicates that the nucleus of each race has compensating factors for the cytoplasm of that race. The same conclusion is also supported by the intermediate position of the homospermic haploid relative to the mean and the heterospermic haploid with the same cytoplasm. Evidently then, the nuclei of the two forms also differ and do so in such a way as to compensate in development for cytoplasmic differences.

Cytoplasmic and nuclear differences seemingly demonstrated, it is of interest to determine which is responsible for the slight dissimilarities between the control diploids, and the more distinct dissimilarities between the homospermic (control) haploids of the two races. It has been shown that each diploid control in its morphogenesis is on the same side of the mean as the hybrid diploid with the same cytoplasm, though not at the same distance. The homospermic haploids, in their development, parallel the mean at a greater distance than the control diploids, suggesting a lesser degree of compensation by the haploid nucleus. The homospermic haploid in its morphogenesis shows the same tendencies, though to a lesser degree, as the heterospermic haploid with the same cytoplasm. These facts suggest that the cytoplasmic differences are responsible for the slight dissimilarities between the diploid controls and homospermic haploid controls of the two forms. Further study may demonstrate whether or not these cytoplasmic differences are also related to the dissimilarities of the two adult parent forms.

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[8] A heterozygous genome in the parent forms could not account for these differences.

What is the nature of these nuclear and cytoplasmic differences? There is not, of course, sufficient information available to answer this question. The presence of some degree of cytoplasmic organization in the amphibian egg has been shown to exist as early as 20 minutes after insemination (Fankhauser, 1930) and before first cleavage (Brachet, 1906), but the nature of this organization has not been demonstrated. Though the differences which are being examined cannot be described in precise terms, one feature of their relative nature does become apparent. It is clear from the results that some property or properties of the cytoplasm of the northern form tend to make the embryos with the cytoplasm of that form display certain features of development which, relative to the mean type representing normal development, are the exact opposite of those found in the embryos with the cytoplasm of the southern form (Table II). This infers that the differences in organization or composition, whether they be quantitative or qualitative, are of opposite natures as measured in terms of what they tend to produce in development. It has been noted further that the nuclei of the two forms have properties which tend to compensate for the cytoplasmic differences. Therefore the nuclei may also be considered to have properties of opposite nature. If this reasoning is correct, it seems that the nucleus of one form should supplement or enhance the cytoplasmic

TABLE II

Northern cytoplasm	Southern cytoplasm
1. *Small* gastrular angle	1. *Large* gastrular angle
2. *Large* completed blastopore	2. *Small* completed blastopore
3. Neural plate abnormally *broad* at anterior end	3. Neural plate abnormally *narrow* at anterior end
4. Neural plate abnormally *short*	4. Neural plate abnormally *long*
5. *Small* tail-bud	5. *Large* tail-bud
6. *Large* head primordia	6. *Small* head primordia
7. *Small* abdomen relative to head size	7. *Large* abdomen relative to head size

influence of the other form. This means that the development of the reciprocal heterospermic haploids should be sufficiently different to suggest the activity of something more than the cytoplasm. While there is no unit of measurement by which the degree of difference can be determined, it is clearly great (Fig. 8) and is probably contributed to by a nuclear influence.

The differences in size of yolk granules and nuclei which preliminary studies have shown to exist between the early embryonic stages of the two forms constitute the only concrete dissimilarities between cytoplasm and nuclei so far observed. What connection, if any, these may have with the actual nuclear and cytoplasmic differences responsible for the above results is not readily apparent.

It is of further interest to determine how these differences operate to produce the results described above. This point is brought into this discussion not because any definite answer can be provided but because certain experimental treatments which could be expected to alter the mode of operation of cytoplasmic and nuclear factors have produced similar results. For example, if a temperature gradient is applied to the developing frog egg, that portion at the warm end of the gradient develops abnormally large structural units (Huxley, 1927; Dean, Shaw, Tazelaar, 1928; Gilchrist, 1928, 1929, 1933). More specifically, if the gradient is applied "adjuvantly" (Huxley, 1927) along the animal-vegetal polar axis in blastula stages (i.e., with warm end of gradient at animal pole) the tail-bud embryos from a blastula so treated have slightly larger heads than the controls and those subjected to the reverse gradient (Huxley and Dean, Shaw, Tazelaar, *loc. cit.*). It is further reported by the same authors that an adjuvant gradient increases by several times the normal difference in size existing between animal and vegetal cells of the blastula stages. Gilchrist (1933) demonstrates that size differences of embryonic structures resulting from temperature gradient treatments are not due solely to age differences but thinks rather that there is an alteration in what he terms the "physiological pattern" of the egg. In this same connection it can be noted that toxic agents applied to developing frog embryos can likewise produce a disproportion of parts most noticeably influencing those regions having the highest metabolic activity at the time of application (Bellamy, 1919).

With these results in mind, it is reasonable to suggest that the differences between the cytoplasms of the eggs of these two geographic forms or races are differences in factors which normally determine the varying rates of metabolism and cell division in the various parts of the developing blastula and possibly the induction processes in later stages. Only one bit of experimental evidence bearing on the physiological properties of these eggs is available and this of a very preliminary sort—the temperature tolerance is higher for the egg of the southern form. This, it is logical to suppose, is related to the fact that the southern embryos may be called upon to develop at higher temperatures than the northern. From this, however, it is not possible to reason that other physiological differences which may exist between the two eggs are likewise related to climatic influences.

It is realized that other subjects of interest could be discussed in relation to the results of these experiments but it is felt that they may be considered more successfully after more information has been accumulated. For the present, it seems best to emphasize that the gametes of two geographic forms probably of the same species differ slightly in their cytoplasmic and nuclear properties and that by androgenetic haploid as well as diploid hybridization the orderly and measurable effects of these properties on early morphogenesis can be observed. The nature of these differences, their mode of operation, the relation of the embryonic differences they produce to the differences between the adults are among the major problems which can be and should be examined later with the same or similar materials and methods.

Summary

1. Two distinct forms of frog, commonly referred to as *Rana pipiens,* Schreber, are described, and evidence is presented to show that they probably represent geographic races of that species, one from northern Vermont, the other from southeastern Pennsylvania.

2. In the experiments described, the gametes of these two races have been combined reciprocally to form diploid and androgenetic haploid hybrids and the early development of these has been studied in detail.

3. The diploid hybrids developed through metamorphosis; the androgenetic hybrids for 7 to 11 days, up to about stage 24 (Shumway, 1940).

4. A comparison of 3-day-old control and hybrid embryos reveals that, in general, the combinations which include cytoplasm of the northern form are characterized by larger head primordia and smaller posterior axial structures than are observed in those with southern cytoplasm. Such dissimilarities, only slightly apparent between the homospermic diploid controls, become progressively more accentuated between the homospermic haploids, the heterospermic (hybrid) diploids, and the heterospermic (hybrid) haploids.

5. A study of gastrula, neurula, and older stages discloses the early expressions and later fate of the dissimilarities shown by the 3-day-old embryos.

6. These results demonstrate:

(*a*) Cytoplasmic differences between the eggs of the two forms which seem to have contrasting effects upon the same developmental processes.

(*b*) Nuclear differences which, in homospermic diploid control development, appear to compensate for the cytoplasmic differences.

(*c*) An orderly cytoplasmic influence on early morphogenesis.

7. The possible nature and mode of action of these differences are briefly discussed.

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THE RELATION BETWEEN HYDROGEN-ION CONCENTRATION AND VOLUME, GEL/SOL RATIO AND ACTION OF THE CONTRACTILE VACUOLE IN AMOEBA PROTEUS [1]

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INTRODUCTION

Von Limbeck (1894) observed that erythrocytes swell if the concentration of carbon dioxide in the blood is increased. Jacobs and Parpart (1931) found that hemolysis in erythrocytes increases with increase in acidity and that the effect of as small a change as 0.01 pH is measurable. Lucké and McCutcheon (1926) maintain that the volume of eggs of Arbacia in sea water does not change with changes in hydrogen-ion concentration between pH 4.2 and pH 9.8 unless the eggs are left so long that they become injured.

Chalkley (1929), in observations on *Amoeba proteus* in balanced salt solution, found that as the hydrogen-ion concentration decreases from pH 6 the volume decreases to a minimum at pH 7 and then increases, i.e. that there are two maxima, one in the acid range and another in the alkaline; and Mast and Prosser (1932) found that as it decreases over the range studied (pH 5.4–8) the gel/sol ratio decreases. Pitts and Mast (1934) investigated the relation between gel/sol ratio and hydrogen-ion concentration in single as well as balanced salt solutions. They confirmed the results obtained by Mast and Prosser and conclude that " in sodium or potassium salt solutions the gel/sol ratio decreases as the hydrogen-ion concentration decreases, but that in calcium salt solutions it increases in the more acid range (pH 5.0 to pH 5.9) then remains constant or decreases slightly."

Thus it will be seen that there is considerable difference of opinion concerning the effect of changes in hydrogen-ion concentration of the medium on the volume of cells, that no observations have been made on *Amoeba* concerning the relation between volume and hydrogen-ion concentration in single salt solutions, and that the results obtained in observations on the gel/sol ratio in these solutions have not been confirmed. It is the purpose of this paper to present detailed information concerning the relation between hydrogen-ion concentration, volume and the gel/sol ratio of *Amoeba proteus* in salt solutions containing respectively sodium, potassium and calcium as the only metallic cations.

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[1] These investigations were carried out under the direction of Professor S. O. Mast in the Zoölogical Laboratory of the Johns Hopkins University and the Marine Biological Laboratory at Woods Hole, Mass. They were greatly facilitated by a grant from the Brooks Fund.

Material and Methods

The amoebae used were selected, prepared, and measured for volume and gel/sol ratio as described in the section on material and methods in a previous paper (Mast and Fowler, 1935). The solutions used consisted of a primary phosphate hydroxide buffer system in which the concentration of the cation was identical in the phosphate and in the hydroxide (Pitts and Mast, 1933). The stock solution of phosphate was kept in a covered Pyrex flask and the stock solution of hydroxide in a carefully sealed Pyrex flask open to the exterior through a soda lime tube and through a 50 cc. Pyrex glass buret. These solutions were standardized according to the method described by Pitts and Mast (1933). By mixing the phosphate and the hydroxide in various proportions the desired hydrogen-ion concentration was easily obtained. The hydrogen-ion concentration of each solution prepared was measured with a quinhydrone electrode and a Leeds Northrup potentiometer.

Volume and Gel/Sol Ratio

Sodium Salts

Ten amoebae were selected, put into modified Ringer solutions,[2] and left for approximately 24 hours. Then the volume and the gel/sol ratio of each were measured as described above, after which they were transferred to a solution containing 0.002 M sodium as the only metallic cation at pH 5.5, left 15 minutes and measured again, after which they were measured at 15-minute intervals for 105 minutes. This was repeated for 60 other individuals, 10 in each of the following solutions: 0.002 M sodium phosphate buffer solutions at pH 6.0, pH 6.5, pH 7, pH 7.5, pH 8.0 and pH 8.8, respectively. There was but little change in either the volume or the gel/sol ratio of the amoebae after they had been in these solutions 30 minutes. All the results obtained in the measurements of volume and gel/sol ratio made at each hydrogen-ion concentration were therefore respectively thrown together and the average calculated. These averages are presented in Fig. 1, *A*.

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[2] 3.3 cc. salt solution (0.35 gram NaCl, 0.14 gram KCl, 0.12 gram $CaCl_2$, 1000 cc. H_2O) + 5 cc. buffer solution (25 cc. 0.2 M KH_2PO_4, 12.5 cc. 0.2 M NaOH, 62.5 cc. H_2O; Clark, 1927) + 91.7 cc. H_2O. This solution is the same in composition as Chalkley's 1/60 Ringer solution (1929). The total concentration of salts is 0.002 M and the hydrogen-ion concentration is pH 6.8.

Figure 1, *A* shows that after the amoebae had been transferred from modified Ringer solution 0.002 M, pH 6.8, to 0.002 M sodium phosphate buffer solutions at the various hydrogen-ion concentrations used, the average volume calculated from measurements made at 15-minute intervals for 105 minutes after transfer, decreased 5 per cent at pH 5.5, 0.3 per cent at pH 6.0, 7 per cent at pH 6.5, 10 per cent at pH 7.0, 7.3 per cent at pH 7.5 and 10 per cent at pH 8.0. (Measurements at

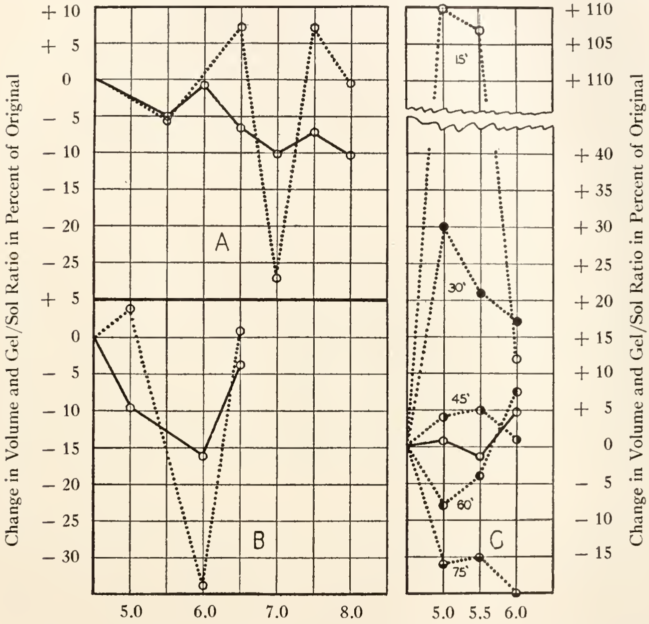

FIG. 1. The relation between volume and gel/sol ratio in *Amoeba proteus* and hydrogen-ion concentration in solutions which contain only one kind of metallic cations. *A,* sodium 0.002 M; *B,* potassium 0.002 M; *C,* calcium 0.005 M; solid curves, volume; broken curves, gel/sol ratio; 0, volume and gel/sol ratio in modified Ringer solution; +, increase in volume and gel/sol ratio; —, decrease in volume and gel/sol ratio; points on curves, averages of 3 to 50 measurements (see text); 15′, 15 min. after transfer from Ringer solution to Ca solution; 30′, 30 min.; 45′, 45 min.; 60′, 60 min.; 75′, 75 min.

pH 8.8 were impossible because the amoebae disintegrated within a few minutes after they had been put into the solutions.)

This indicates that as the hydrogen-ion concentration in a sodium solution decreases from pH 5.5 the volume increases to a maximum at pH 6, then decreases to a minimum at pH 7, then increases to a secondary maximum at pH 7.5, and then decreases again.

Figure 1, *A* also shows that the gel/sol ratio decreased 5.5 per cent at pH 5.5, 27 per cent at pH 7 and zero per cent at pH 8, and that it increased 7.5 per cent at pH 6.5 and pH 7.5. This indicates that in a sodium solution as the hydrogen-ion concentration decreases from pH 5.5 the gel/sol ratio increases to a maximum at pH 6.5, then decreases rapidly and very greatly to a minimum at pH 7, then increases equally rapidly and greatly to a second maximum at pH 7.5 and then decreases again.

Potassium Salts

The experiments concerning the relation between volume and gel/sol ratio and hydrogen-ion concentration in solutions containing potassium as the only metallic cation were performed the same as those containing only sodium, except that three specimens were used for each hydrogen-ion concentration in place of ten. The results obtained show that there was, as in the sodium solutions, but little change in volume and gel/sol ratio after the amoebae had been in the solutions 15 minutes. The averages of all the results obtained concerning volume and gel/sol ratio were therefore respectively calculated. These averages are presented in Fig. 1, *B*.

Figure 1, *B* shows that in amoebae transferred from modified Ringer solution, pH 6.8, to potassium phosphate buffer solutions at various hydrogen-ion concentrations, the volume decreased 10 per cent at pH 5.0, 16.1 at pH 6.0, and 3.7 at pH 6.5, and that the gel/sol ratio increased 4 per cent at pH 5 and about 1 at pH 6.5 and decreased 33.8 per cent at pH 6. In neutral and alkaline solutions the amoebae disintegrated so rapidly that it was impossible to measure them.

These results indicate that as the hydrogen-ion concentration in potassium solutions decreases from pH 5 the volume of *Amoeba proteus* decreases slowly to a minimum at pH 6 and then increases rapidly; and that the gel/sol ratio decreases very rapidly to a minimum at pH 6 and then increases equally rapidly.

Calcium Salts

The methods used in the observations on amoebae in solutions in which calcium was the only metallic cation present are the same as those used in the preceding experiments. The concentration of calcium was 0.005 M and ten amoebae were measured at each of four hydrogen-ion concentrations: pH 5, 5.5, and 6. In lower hydrogen-ion concentrations, it was impossible to maintain the concentrations long enough to make the measurements.

The results obtained show that there was but little change in volume after the amoebae had been in the solutions 15 minutes, but that the gel/sol ratio changes radically with time. In reference to volume, the average for all the measurements made at each hydrogen-ion concentration was therefore calculated; but in reference to gel/sol ratio the average of the results obtained in the measurements made after each 15-minute period at each hydrogen-ion concentration was calculated. These averages are presented in Fig. 1, *C*.

Figure 1, *C* shows that in the amoebae which were transferred from modified Ringer solution pH 6.8 to calcium phosphate buffer solutions the volume increased 0.8 per cent at pH 5 and 4.8 per cent at pH 6 and that it decreased 1.4 per cent at pH 5.5. This indicates that as the hydrogen-ion in calcium solutions decreases from pH 5 the volume decreases slightly to a minimum at pH 5.5 and then increases fairly rapidly.

This figure shows that during the first 15 minutes after the amoebae had been transferred from modified Ringer solution the gel/sol ratio increased 110 per cent at pH 5.5, 107 per cent at pH 6.5 and 12 per cent at pH 6, and that it then decreased during the following 90 minutes to 16 per cent below the original ratio at pH 5, 15 per cent at pH 5.5 and 20 per cent at pH 6.

This indicates that after transfer from Ringer solution to calcium solution the gel/sol ratio increases very rapidly and very extensively, if the hydrogen-ion concentration is relatively high, and then gradually decreases and that the extent of the change in this ratio varies directly with the hydrogen-ion concentration.

The Action of the Contractile Vacuole

The results presented by Chalkley (1929) and those presented in the preceding pages show that the volume of *Amoeba proteus* is correlated with the hydrogen-ion concentration of the surrounding medium. The question now arises as to whether or not this correlation is dependent upon the action of the contractile vacuole. This problem was investigated as follows.

Fifty to one hundred amoebae were transferred successively through three beakers each containing 50 cc. redistilled water and left in the last for one hour. During this time many of the amoebae became radiate in form. About 25 of these were selected and put into 50 cc. 0.002 M Ringer solution (pH 6.8) and left 12–15 hours, then an actively moving specimen was selected and measured in the volumescope as previously described (Mast and Fowler, 1935). It was then transferred to test solution, in the depression on a Pyrex glass slide, and covered with a cover-glass, after which the diameter of the vacuole, immediately preceding contraction, and the interval between successive contractions were measured with a Filar micrometer ocular and with a stop watch respectively. This was continued as long as desired, after which the whole process was repeated with other individuals in this and in other solutions. Then the average volume of fluid eliminated by the contractile vacuole per amoeba per minute and the average volume eliminated per minute in percentage of the volume of the amoebae were calculated for each solution used.

There was considerable variation in given amoebae during the period of observation in the different solutions used and in the intervals between successive contractions, but these variations were not specifically correlated with time in any of the solutions except the Ringer-lactose solution, a solution in which the osmotic concentration was relatively very high. In this solution the size of the vacuole decreased and the interval between contractions increased with time and there usually were not more than five contractions before it ceased to function altogether. These statements are substantiated by the following typical results.

In one of the amoebae transferred to Na buffer solution (pH 6.5), the diameter of the first vacuole in the series of ten measured was 2.36 μ, that of the last 2.30 μ, that of the smallest 2.07 μ and that of the largest 2.66 μ.

In one of the amoebae transferred to the Ringer-lactose solution, the diameter of the first vacuole was 2.42 μ and those of the following three were 2.79 μ, 2.69 μ and 2.38 μ respectively, and the intervals between the successive contractions in the series were 2′ 45″, 3′ 5″, 5′ 0″ and 6′ 30″. After this series of contractions was complete the vacuole was continuously observed for 45 minutes. It did not contract during this time but it became smaller, the diameter at the end of three successive 15-minute intervals having been 1.75 μ, 1.66 μ and 1.57 μ respectively. In this amoeba the vacuole ceased contracting 17 minutes and 20 seconds after transfer to the Ringer-lactose solution. The average time required for cessation of contraction in this solution was 18.5 minutes.

The averages of the results obtained directly by observation of amoebae in the different solutions used and those obtained by calculations are presented in Table I.

This table shows that the size of the contractile vacuoles in the amoebae in all the different solutions used except the Ringer-lactose solution and the Na buffer (pH 8) was essentially the same.

In the Ringer-lactose solution the vacuole decreased markedly with time, as stated above, hence the low average diameter of 2.09 μ. The high average diameter of 2.76 μ in the Na buffer (pH 8) appears to have been directly correlated with the size of the amoebae in this solution.

TABLE I

The volume of fluid eliminated by the contractile vacuole in Amoeba proteus *in various solutions.* Temperature, 25° C. Ringer and Na buffer solutions are described in the text. The volume of only three of the nine amoebae in pure water was measured. The average volume for these is 4980 cμ and the average elimination 0.30 per cent of this volume.

Solutions used	Amoebae studied		Contractile vacuoles				
	Number	Av. vol. in 1000 cμ	Number measured	Av. diam. in μ	Av. interval between contractions	Av. vol. of fluid eliminated in cμ per min. per amoeba	Av. percentage of vol. of amoebae eliminated per min.
Ringer 0.002 M pH 6.8	10	1785	100	2.46	3′ 49″	5670	0.31
Ringer 0.002 M pH 6.8 −0.2 M lactose	10	?	42	2.09	9′ 47″	1500	0.008
Na buffer 0.002 M pH 5	5	1753	50	2.29	4′ 5″	4260	0.24
Na buffer 0.002 M pH 6	6	1891	60	2.25	4′ 10″	4620	0.24
Na buffer 0.002 M pH 6.5	6	1619	60	2.22	4′ 4″	3900	0.24
Na buffer 0.002 M pH 7	5	2184	50	2.43	5′ 31″	7380	0.17
Na buffer 0.002 M pH 8	10	3141	100	2.76	4′ 2″	7620	0.24
Pure water	9	1625	90	2.61	3′ 36″	4980	0.30

The table shows that the average interval between successive contractions was relatively small in pure water and Ringer solution and considerably higher but essentially the same in all the Na buffer solutions except pH 7 in which it was relatively very high. It shows that the rate of elimination per unit volume of protoplasm was relatively high in pure water and Ringer solution and considerably lower but essentially the same in all the Na buffer solutions except pH 7, in which it was much lower.

The results obtained consequently indicate that the rate of elimination of fluid by the contractile vacuole in *Amoeba proteus* is practically independent of the hydrogen-ion concentration except in the region of neutrality where it decreases markedly and that it gradually decreases to zero in hypertonic solutions.

Discussion

The results presented in Table I show that in amoebae which have been transferred from Ringer solution to sodium solution of various hydrogen-ion concentrations, there was a change in the rate of elimination of fluid by the contractile vacuole of only 0.07 per cent of the volume of the amoebae per minute. It is consequently obvious that the action of the contractile vacuole was only slightly involved in the changes in the volume of the amoebae observed, in relation to changes in hydrogen-ion concentration (Fig. 1), and that these changes were consequently largely due to the effect of the hydrogen-ion concentration of the solutions used, on the permeability of the surface layer to water.

Table I and other evidence presented above show that in the amoebae which had been transferred from 0.002 M Ringer solution to 0.002 M Ringer solution plus 0.2 M lactose, the rate of elimination of fluid by the contractile vacuole decreased from 0.3 per cent of the volume of the amoebae per minute to zero in an average of 18.5 minutes. No observations were made on the action of the vacuole in amoebae which had been transferred in the opposite direction, but it is highly probable that after such a transfer the vacuole becomes active as rapidly and to the same extent as it becomes inactive after the reverse transfer. If this is true, decrease in volume of amoebae in hypertonic solution is considerably augmented and increase in volume of amoebae in hypotonic solution is considerably retarded, owing to elimination of fluid by the vacuole; that is, the amount of fluid which leaves the amoebae directly through the surface in the hypertonic solution is equal to the increase in volume minus the amount eliminated by the vacuoles and the amount which enters the amoebae directly through the surface in the hypotonic solutions is equal to the increase in the volume of the amoebae plus the amount eliminated by the vacuoles.

Mast and Fowler (1935) calculated the permeability constant for water from results obtained in observations on the increase in the volume of amoebae in hypotonic solutions, but they did not consider the effect of the action of the contractile vacuole on the volume. As stated above, this probably amounted to 0.3 per cent of the volume of the amoebae per minute soon after the transfer to these solutions. The calculated value obtained by them (0.026) is therefore somewhat too small.

In the preceding paper it was demonstrated that after amoebae have been transferred from Ringer solution to this solution plus 0.2 M lactose, they decrease about 15 per cent in volume in 15 minutes and it was demonstrated above that under these conditions they continue to contract for about 18.5 minutes. It is therefore obvious that elimination of fluid through the vacuole continues after considerable fluid has passed out of the body by diffusion and that the action of the vacuole is not immediately dependent upon entrance of fluid and turgidity of the cell. This also obtains for other protozoa (Kitching, 1938, p. 148).

Pitts and Mast (1934), in observations on the gel/sol ratio in *Amoeba proteus*, obtained results which in general support the conclusion reached above, namely that the gel/sol ratio is relatively low in the region of neutrality. They also found that the rate of locomotion is low in this region and Table I above shows that the rate of elimination of fluid by the contractile vacuole is also low in this region. This indicates that the rate of locomotion and the action of the vacuole vary inversely with the fluidity of the cytoplasm and that it is maximum at neutrality. This is probably in some way correlated with the isoelectric point of a prominent protein in the cytoplasm.

The extraordinary changes observed in the gel/sol ratio in calcium solutions indicate remarkably rapid and extensive adjustment but concerning the processes in this adjustment there is no evidence.

Summary

1. As the hydrogen-ion concentration decreases from pH 5.5 the volume of *Amoeba proteus* in solutions containing sodium as the only metallic ion increases to a maximum at pH 6.0, then decreases to a minimum at pH 7.0, and then increases to a second maximum at pH 7.5; and the gel/sol ratio increases to a maximum at pH 6.5, then decreases very extensively to a minimum at pH 7.0, and then increases equally extensively to a second maximum at pH 7.5.

2. In solutions containing potassium as the only metallic ion the volume and the gel/sol ratio decrease to a minimum at pH 6.0 and then increase.

3. In solutions containing calcium as the only metallic ion the volume remains nearly constant, but the gel/sol ratio increases very rapidly and extensively and then gradually decreases; but the extent of change in this ratio varies directly with the hydrogen-ion concentration.

4. The rate of elimination of fluid by the contractile vacuole is practically independent of the hydrogen-ion concentration except in the region of neutrality where it decreases markedly. In hypertonic solutions it gradually decreases to zero.

5. The change in the rate of elimination of fluid by the vacuole in relation to hydrogen-ion concentration is so low in comparison with the change in rate of passage of fluid directly through the surface that it is negligible. The changes observed in the volume of the amoebae in relation to the hydrogen-ion concentration were therefore almost entirely due to changes in the rate of transfer of fluid directly through the surface, i.e. to changes in the permeability of the surface to water.

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PIGMENT MIGRATION IN THE EYES OF THE MOTH, EPHESTIA KUEHNIELLA ZELLER

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Introduction

The movement of pigment in the eyes of insects has been described many times, but there is little information in the literature suggesting what factors are involved in bringing about this movement. In certain Crustacea it has been conclusively demonstrated (Kleinholz, 1936, 1938; Welsh, 1939, 1941) that the movement of the pigment from the " dark " position into the " light " position is dependent upon the action of a hormone secreted by the eye stalk. In view of recent discoveries of reciprocal effects between insect and crustacean hormones (Hanström, 1937; Brown and Meglitsch, 1940), the question arises as to whether endocrines also regulate pigment migration in the moth eye. There are at least three ways in which eye pigment migration in an insect might be controlled, namely, by hormones, by nerves, and by the action of the pigment cells as independent effectors. Possibly also a combination of these three methods of control might be found in some insects.

Many of the early workers (see Demoll, 1911) believed that the control was nervous in nature. But the experiments of Exner and Demoll (*cf.* review by Parker, 1932), and the subsequent work of Uchida (1934) on the long-horned grasshopper, indicated that the movement was generally slow in comparison with most nervous responses. Friza (1928) suggested, as a result of his study of *Mantis religiosa,* that the movement was humorally controlled. In addition, the work of Horstmann (1935), in which he corroborated the conclusions of the older workers on the existence of a diurnal rhythm in pigment migration under constant conditions of the external environment, indicates that the response is not that of independent effectors, in which the pigment cells respond directly to light. Collins (1934), however, concluded that it was most likely that light acted directly on the pigment cells of the moth, *Carpocapsa.*

In the following experiments, the movement of the eye pigment of the moth, *Ephestia kuehniella* Zeller, will be described, and an attempt made to determine which of the three above-mentioned factors is responsible for the movement. The study is based on the examination of serial sections of the heads of over 450 moths.

The moths were bred in large glass jars, each containing about two inches of dry oatmeal. Under these conditions the life-cycle occupied about six weeks, a plentiful supply of adult moths thus being available at all times. Observations were made on sections of entire heads fixed in alcoholic Bouin's fluid, which penetrated with sufficient rapidity that no migration of pigment occurred. Results were comparable in this regard with those obtained with fixation by hot water, but histological preservation was better. Some sections were stained in Mallory's triple stain or impregnated by Bodian's protargol method, and others were depigmented in Grenacher's fluid, and subsequently stained or impregnated.

It is a pleasure to thank Professor J. H. Welsh and Dr. L. H. Kleinholz for suggestions during the course of this work.

Anatomical Considerations

Umbach (1934) described the eye structure of *Ephestia* in detail. The following description will therefore deal only with the pigment cells and other points which directly concern our discussion. Features of a single ommatidium are shown in Fig. 1.

The pigment in the eye is arranged in three distinct groups, but attempts to homologize these with the pigment cells of Crustacea were unsuccessful. One group of pigment granules surrounds the bases of the rhabdoms and also extends below the basement membrane. Umbach (1934) has shown that this pigment in *Ephestia* is not contained in specific retinal pigment cells, as it is in many insects. Although in *Ephestia* the pigment is never completely withdrawn beneath the basement membrane, as in *Vanessa* (Demoll, 1917), it apparently exhibits some movement. But the extent of this movement does not exceed ten microns, and no attempt has been made to determine its cause.

A second group of pigment granules is contained in cells which have a varied terminology. They are here called primary pigment cells, but have been referred to as iris pigment cells by Snodgrass (1935), as primary iris cells by Wigglesworth (1939), distal pigment cells by Collins, and accessory pigment cells by Uchida. These cells closely surround the crystalline cones. The nuclei are extremely flattened, and the pigment granules are arranged in a thin layer around the cones. Neither these cells nor their pigment granules have been observed to migrate. Finally, the large accessory pigment cells (secondary iris cells, Wigglesworth; principal pigment cells, Uchida) are very conspicuous and contain most of the pigment granules. The entire cell moves, as well as the pigment granules contained in it, for the nuclei have been shown in depigmented preparations to have moved through a distance of about 30 microns, with the mid-point at about the proximal end of the crystalline cone. None of the pigment in the eyes of *Ephestia* has a reflecting function.

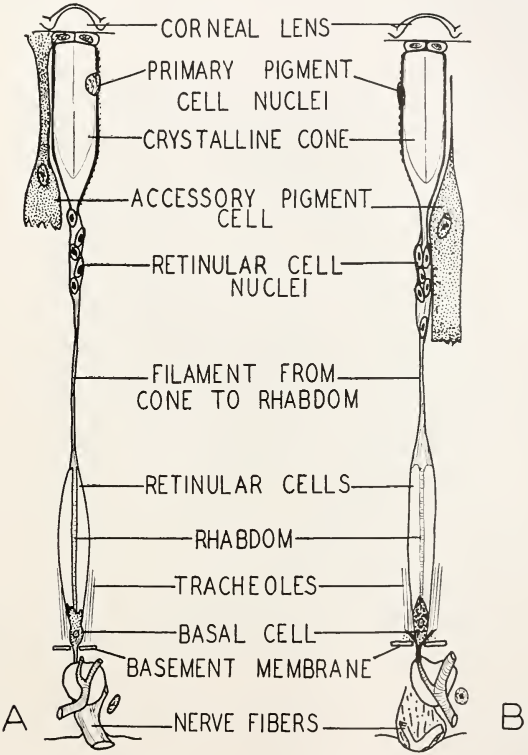
CORNEAL LENS
PRIMARY PIGMENT CELL NUCLEI
CRYSTALLINE CONE
ACCESSORY PIGMENT CELL
RETINULAR CELL NUCLEI
FILAMENT FROM CONE TO RHABDOM
RETINULAR CELLS
RHABDOM
TRACHEOLES
BASAL CELL
BASEMENT MEMBRANE
A
NERVE FIBERS
B

FIG. 1. Longitudinal sections of two ommatidia from eyes of *Ephestia* in (*a*) dark-adapted, and (*b*) light-adapted positions, respectively.

Another important aspect of the structure of the eye is the size of the ommatidia. Umbach (1934) states that there are between 2,000 and 2,500 in each eye. Between 60 and 70 of these are seen in each eye in a transverse section of the head (Fig. 2). The ommatidia vary

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in size, the smallest being on the dorsal side, and the largest on the ventral side of the eye, the difference between the two extremes being about 20 per cent of their length. The accessory pigment cells migrate a greater distance in the larger ommatidia than they do in the smaller ones, as in Crustacea, where the greatest amount of migration occurs in the largest ommatidia which are found on the dorsal side of the eye.

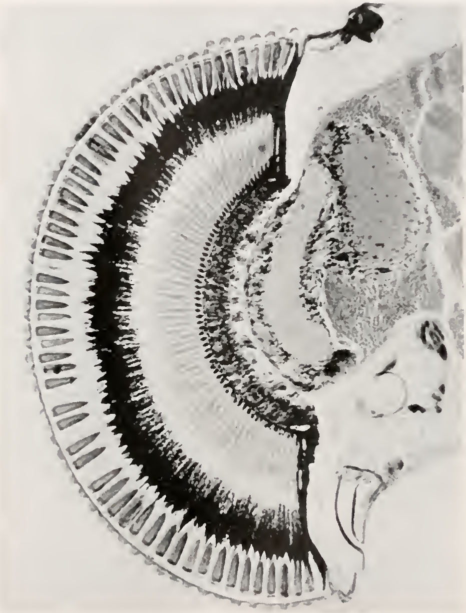


FIG. 2. Transverse section of part of head of *Ephestia,* showing the arrangement and differences in size of the ommatidia. Pigment cells in light-adapted position.

These differences make it necessary to select the central ommatidia for comparisons between eyes of insects treated in different ways. Also, the eyes of female moths are on the average slightly larger than those of males. The average length of the central ommatidia of ten males, measured from the basement membrane to the distal end of the crystalline cone, was 177.3 microns, while that from ten females was 186.8 microns, longer than the average of the males by 5 per cent. Therefore insects of only one sex were used in experiments when they were to be directly compared.

Two other details of anatomy should be mentioned. The first is that no contractile fibers, such as were demonstrated by Welsh (1930) in the eyes of *Palaemonetes,* have been seen in the eyes of *Ephestia.* Movement of pigment granules and of the pigment cells probably results, as was suggested by Bennitt (1924), and Parker (1932), from protoplasmic streamings or surgings, and a similar mechanism will probably explain the interesting movement (first demonstrated by Umbach) of the retinular cells themselves. Finally, Bodian preparations of both pigmented and depigmented eyes showed no sign of effector nerves supplying the accessory pigment cells. The significance of such negative evidence is, however, questionable in view of the difficulty of demonstrating nerves to sense hairs which occur in large numbers on the eyes of some insects.

Normal Movement of the Accessory Pigment Cells

The normal movement of the accessory pigment cells, such as occurs when a dark-adapted moth is exposed to light, can be followed by referring to Fig. 3. First, it is to be noticed that the extreme dark position, as seen in *A,* is rarely found. Moths kept for several days in the dark more frequently have their accessory pigment cells in a position similar to that in *B.* In this stage pigment granules are evenly distributed and extend from the level of the distal ends of the cones to about 15 microns beneath their proximal ends, the level of the grouped retinular cell nuclei. The first movement of pigment in such an eye usually becomes evident as an increasing aggregation of the granules in the proximal part of the cells. This region of the cell then moves proximally toward the basement membrane (Fig. 3, *D*), accompanied by a simultaneous decrease in the size of the extensions of the pigment cells between the cones. As the proximal movement continues these extensions become attenuated (Fig. 3, *E*), and the characteristic " frayed " region at the distal end is eventually withdrawn (Fig. 3, *F*). Between the two last-mentioned stages, movement of the pigment cell nuclei occurs. The final stage of movement involves the almost complete withdrawal of granules of the accessory pigment cells from between the cones (Fig. 3, *G*). Light alone is incapable of causing greater proximal movement than that seen in Fig. 3, *G,* but certain treatments described below may induce more extensive movement.

The movement in *Ephestia* is not so simple a migration as was indicated by Collins (1934, Pl. 4) in *Carpocapsa.* Measurements of the distance of the pigment cells from the cones do not indicate all the changes that occur, since two types of movement, lateral as well as longitudinal, are found. These are seen in stages *E* and *F*, and are equivalent to those observed by Peabody (1939) in the isopod, *Idothea*. The fact, well known in Crustacea, that the type of pigment migration varies in different species, holds also for insects.

EXPERIMENTAL RESULTS

Diurnal Rhythm

The first experiments were made to determine whether a persistent diurnal rhythm existed in the movement of the pigment cells, as previously recorded for many insects and Crustacea (for review, see Welsh, 1939). In *Ephestia,* kept under constant illumination for four days, the pigment cells were found in the light-adapted position at any hour of the day or night. Likewise, insects kept in darkness, with all other usually controlled factors maintained constant, had pigment cells in the dark position during both day and night. This is believed to be the first reported absence of a persistent diurnal rhythm in a moth. However, in view of the marked interspecific differences recorded by Welsh (1935), even within a single genus of crustaceans, and the absence of diurnal rhythm of pigment migration in *Palaemonetes* (Kleinholz, 1936), the differences between various insects are perhaps not so surprising.

The majority of previous workers on Lepidoptera have made their observations on the changes of the pseudopupil, or on the presence of glow in the eye when illuminated at night. It has rarely been possible to demonstrate glow during the day. In *Ephestia* in constant darkness the eyes glow during the day as well as at night. The glow, however, disappears rapidly upon illumination,—as shown for other insects by many observers (see Merker, 1929). At a light intensity of 12 ft-candles, 1 to 2 minutes are required for the glow to disappear from dark-adapted *Ephestia* at 25° C. Sections of eyes which exhibited glow and those from which it had just disappeared show that the changes in position of the pigment cells are frequently almost imperceptible. Glow in *Ephestia* is not caused by reflecting pigment, as in some Crustacea, nor does it seem likely that its appearance is due to the reflection of light from the tracheal tapetum between the rhabdoms. The cause of glow in insects deserves further study.

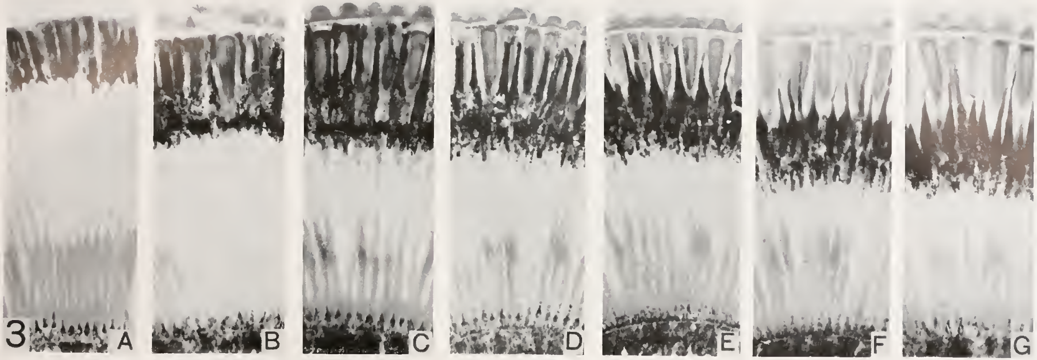
The Effect of Light

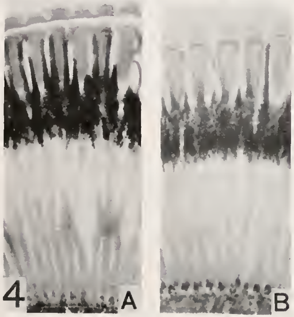
Intensity.—The effect of light of various intensities was determined. A standard source (15-watt Mazda bulb, filtered through Wratten Neutral Filter) was maintained at a constant distance from a group of insects which were inclosed, as in all subsequent experiments, in thin, 2-dram glass vials, plugged with cotton wool, and standing, bottom uppermost, in a container with white walls. All experiments were begun with an excess of moths, and insects which were persistently active, or which were settled facing away from the light, were not used. It was soon found that several factors had to be controlled in order to obtain consistent results. Since it seemed that the rate of response was influenced by the age of the moths, only newly emerged virgins were used. A further complication was found in some moths due to the presence of a parasite. In such cases many spores were found in sections of the head, usually in the hemocoele, but sometimes also in the tissues. Such individuals were not considered in the experiments.

Light intensity was varied by accurately calibrated neutral filters. The results are indicated in Fig. 4. In this method of presenting data, the technique employed involves measuring the pigment migration of a number of individuals (in this case, five), and then photographing an eye whose measurement is nearest the average. After 30 minutes exposure to an intensity of approximately 0.3 ft-candles, migration into the light position is incomplete. Above 3 ft-candles a maximum response is produced in the same period of time. While these results are readily reproducible, single measurements of proximal migration are insufficient to permit a determination of the exact type of relationship between intensity of light and distance migrated. In all subsequent experiments an intensity of 12 ft-candles was employed.

The Rate of Movement.—The next step was to determine the time taken for the migration of the pigment cells in both directions, when intensity, temperature, and other factors were kept constant. The only method which is available in the case of *Ephestia* is to fix and section eyes of moths which have been exposed to light for varying periods of time. Since this method has been shown by Welsh (1930) to be unsatisfactory in comparison with methods of direct observation which are applicable to animals with stalked eyes, the times given below can only be considered to indicate the approximate rates.

The results of one experiment are recorded in Fig. 3 (*B–G*). Under the conditions of the experiment, movement could be demonstrated within one and one-half minutes after the exposure of the moths to light. Movement was most rapid between 4 and 7 minutes, and was completed within approximately 12 minutes. If, after 30 minutes, the light is removed, dark adaptation begins within 5 minutes, but takes somewhat longer for completion than movement under the influence of light, a conclusion in agreement with that of all previous workers on Crustacea and insects. In these experiments movement back to the dark position required about 20 minutes.





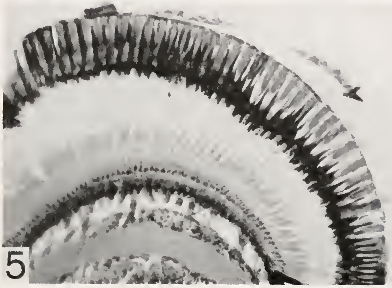


PLATE I

3. Movement of accessory pigment cells in the eyes of *Ephestia* on exposure to light. (*A*) Extreme dark position, (*B*) Usual dark position, (*C*) 0.5 min. light, (*D*) 3.0 min. light, (*E*) 5.0 min. light, (*F*) 8.0 min. light, (*G*) 10.0 min. light.

4. The effect of light intensity. (*A*) 0.3 ft-candles, (*B*) 3.0 ft-candles.

5. Movement of only those pigment cells associated with illuminated ommatidia, when most of the eye is covered by an opaque mixture.

The Effect of Localized Light.—In the experiments of Bennitt (1932) exposure of only a small region of one eye of a crayfish resulted in pigment migration in all ommatidia of that eye and of the other eye as well. Comparable experiments were performed with etherized *Ephestia* by covering both eyes except for a few ommatidia of one eye with the opaque mixture recommended by Crozier, Wolf, and Zerrahn-Wolf (1937). These moths were allowed to recover in the dark, and after 24 hours were exposed to 12 ft-candles for 20 minutes. Under these conditions the sectioned eyes frequently showed that only the region which had been exposed to the light exhibited pigment migration (Fig. 5). Bennitt's results would be expected on the basis of the humoral theory of control of migration. The ease with which it is possible to obtain unequal movement of different pigment cells in the same eye in *Ephestia* suggests that the humoral theory of control may not be applicable in this case. That other insects may react in a manner similar to *Ephestia* has been indicated by the work of Exner (1891) and Demoll (1911).

The Effect of Temperature

Rate of Migration.—Experiments exactly simulating those reported above on the rate of migration under constant light intensity were performed at a temperature of 12.5° C. instead of at 25° C. It was found that in the dark the pigment cells were extended over a greater distance proximally at 12.5° C. than at 25° C. (Fig. 6). However, movement of the pigment cells during light adaptation was in no way different at the lower temperature, except that they did not have so far to travel before reaching the light position. The indications are, then, that the processes of movement have a temperature coefficient of 1.0 within the range 12.5 to 25° C. (but compare the results of Bennitt (1924) on *Gammarus*).

Extremes of Temperature.—When the factor of temperature was found to have an effect on the position of the pigment cells in darkness, a series of experiments was performed to determine the effects of more extreme temperatures. Moths were placed in light-tight containers at temperatures of 3°, 5°, 10°, 12.5°, 25°, and 37° C. for two hours. The moths at the lowest temperature became immobile and those at 37° were extremely active. All were living, however, and were fixed in the dark. Sections showed that the eyes of moths at temperatures from 10° to 25° were in the normal dark-adapted position. At 3°, however, there was considerable movement toward the light position, while at 37° the pigment cells were concentrated between the cones, thus showing a more complete dark adaptation than is ever found under normal temperatures (Fig. 7). This demonstrates for *Ephestia* a conclusion reached long ago for Crustacea by Congdon (1907), that low temperature produces the same effect as light, and that high temperature has an opposite effect. In view of this latter finding, the result of two mutually opposite stimuli was investigated by exposing moths to a temperature of 37° C. and a light intensity of 12 ft-candles at the same time. Under these conditions the effect of light predominated over the effect of heat in nine cases. However, in one moth, where the thresholds must have been approximately equalized, some of the pigment cells migrated into the extreme light position, while others migrated only slightly (Fig. 8). Interestingly enough, corresponding ommatidia in both eyes exhibited the same response, suggesting the possibility of a central control.

The amount of proximal migration produced in the dark by low temperature is greater in the ommatidia on the ventral side of the eye than in those on the dorsal side, as is the case in light adaptation at normal temperatures. The suggestion that such differences in the light-adapted eye are due to differences in the amount of light reaching each ommatidium is therefore untenable.

The Effect of Mechanical Stimulation

The rather unexpected effect of shaking which Horstmann (1935) reported on the phototactic responses of moths has been substantiated with *Ephestia*. If a vial containing a moth is held toward the light and is tapped lightly, the moth exhibits a marked negative response. If the tapping is continued for about 15 seconds the moth will suddenly turn, progress towards the light, and is thereafter strongly positively phototactic. Sections were made of both light and dark-adapted *Ephestia*

PLATE II

Explanation of Figures

6. The effect of temperature, showing spreading of pigment granules after 1 min. in light at 12.5° C.

7. The effect of (*A*) darkness and 3° C., (*B*) light and 37° C.

8. Irregular movement of pigment cells induced by simultaneous action of high temperature (37° C.) and light (12 ft-candles).

9. The effect of anaesthetization by ether. Note the clumping of pigment granules.

10. The effect of the injection of chloretone, inducing greater movement into the light position than is ever produced by light alone.

11. The irregular movement into the light position induced by high tensions of carbon dioxide.

12. Transverse section of the head, illustrating the effect of severing the left optic tract. Pigment in the left eye is in the light position, while that in the eye on the uninjured side is in the dark position.

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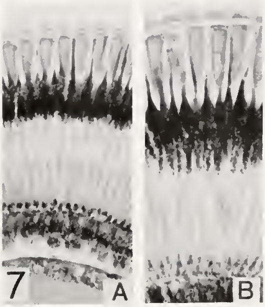
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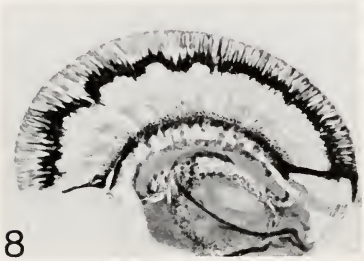
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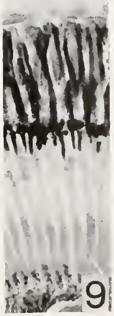
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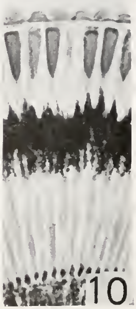
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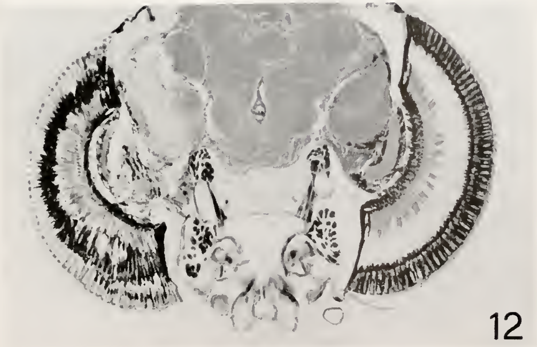
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PLATE II

before and after shaking, and of those which were positively and negatively phototactic. It was found that shaking had no demonstrable effect on the position of the accessory pigment cells, nor was there any effect of shaking on the rate of disappearance of glow. Conversely, glow could not be made to reappear just after its disappearance by any kind of mechanical stimulation. We can only conclude that in the case of *Ephestia* differences in phototactic response are not necessarily correlated with obvious changes in the position of the pigment cells.

The Effects of Certain Drugs, Anaesthetics, and Extracts

Methods.—Injections of approximately 0.001 ml. of fluid were made into the thorax of moths by the microinjection method of Ephrussi and Beadle (1936). Anaesthetization and injection, which occupied between one and two minutes, were performed in dim light. The moths were then kept 15 to 20 minutes in darkness before fixing the eyes. Since it was possible that some of the injected substances might cause movement into the dark position, some of the injected animals were exposed to light.

Ether.—In concentrations just sufficient to anaesthetize a moth, ether has the effect of sending the pigment even further into the dark position than dark alone. A secondary effect is noted when moths are exposed to ether for longer periods of time. The pigment cells are then apparently disorganized, and have a slight tendency even to move proximally in an irregular fashion. The pigment granules may assume a clumped appearance (Fig. 9). The illumination of moths which are under the influence of either of these effects results in apparently normal proximal movement.

Chloretone.—Injection of substances causing proximal migration of pigment cells will obscure the effects of ether. When a saturated aqueous solution of chloretone is injected into a dark-adapted moth, the pigment cells will migrate into the light position, even though the moths are kept in the dark. Usually the movement will be comparable to that produced by light, but sometimes the distance migrated is greater than is ever produced by light alone (Fig. 10). Chloretone might inhibit nerve impulses which would result in maintaining the pigment in the dark position. This inhibition could be exerted either upon motor nerves or on nerves innervating an incretory organ. A similar criticism can be made of any experiments with anaesthetics or drugs.

The Effects of Adrenalin, Acetylcholine, and Prostigmin.—If movement of pigment cells is under the control of the nervous system, it might be expected that the application of appropriate chemical mediators would result in their response. Adrenalin and acetylcholine have both been extracted from insects (von der Wense, 1938; Corteggiani and Serfaty, 1939) but the existence of an adrenergic or cholinergic system has not been proved. The injection, with appropriate controls, of dilutions of 1:1,000 and 1:10,000 adrenalin, and 1:10,000 and 1:100,000 acetylcholine produced no change in the position of the pigment cells. Prostigmin, though having some of the effects on behavior of *Ephestia* comparable to those described for eserine in the mantis by Roeder (1939), did not influence the pigment cells.

Head Extract.—Experiments comparable to certain of those of Kleinholz (1936) were performed on *Ephestia*. The heads of ten light-adapted moths were triturated in 1 ml. of insect Ringer. The extract was boiled, cooled, and injected in the manner described above. Neither this concentration of the extract, nor a dilution of one part of extract to five of Ringer, produced any proximal migration of the pigment cells when the insects were kept in the dark. Nor did such injections inhibit movement when they were exposed to light.

Sinus Gland Extract.—The sinus glands of three specimens of *Uca pugilator* were ground in 0.5 ml. of insect Ringer. The extract was boiled, cooled, and injected into dark-adapted moths. The results were negative, as with extracts of *Ephestia*.

The Effect of High Tensions of Carbon Dioxide.—The striking anaesthetic effect of carbon dioxide on insects has been known for a long time. In *Ephestia* carbon dioxide produces a proximal migration greater than is ever produced by light alone. But this movement is not uniform, so that all cells do not react to exactly the same extent (Fig. 11). The effect on the position of the pigment is still marked after 20 minutes in the dark, by which time the insects have completely recovered from the anaesthetic effects. Bennitt and Merrick (1932) have reported a comparable result in Crustacea due to crowding, which they associate with oxygen lack.

The Effects of Operative Techniques

With carefully sharpened No. 12 hard steel needles an incision was made in the head capsule on the side of the frons along the ocular suture where the cuticle is easily punctured. From this approach the optic tract can be severed. Twenty moths were successfully operated upon in this way, and lived at least four days thereafter. It is of interest to note that an operation of this kind apparently releases the female from certain inhibitions to oviposition, for eggs were deposited in the vials far more frequently by operated than by control females. Several operations did not completely sever the optic tract, and the results of such cases constitute controls. In every case in which the nerve fibers were completely severed, the pigment in that eye was in the light-adapted position, irrespective of the position of the pigment in the eye on the uninjured side. Moreover, once the pigment was brought into the light position by cutting the optic tract, it could never be caused to migrate back to the dark position, although appropriate methods (heat, ether, dark, etc.) were employed. Thus Fig. 12 shows a section of the head of an insect which had been kept for four days in the dark after an operation had been performed on the left eye (left side of the illustration). The pigment cells on the unoperated side occupy the extreme dark position, while those on the operated side have taken up positions characteristic of cells released from their usual control, i.e., they have migrated further proximally than they would under normal light conditions, and their movement is somewhat irregular. The difference between two such eyes can be detected in the living moth.

Discussion

Two of the above experiments provide strong evidence against a hormonal control of movement. The effect of short exposures of light on a small number of ommatidia results in the movement of only a few pigment cells. A hormone in the blood stream would be more general in its action. But this experiment does not differentiate between a nervous control and the possibility that the cells behave as independent effectors. This latter possibility is shown to be untenable, however, by the results of cutting the optic tract, which also provides further evidence against the theory of humoral control, since the blood supply to the eye on the injured side is in no way impaired. There remains only the theory of the nervous control of pigment migration.

The absence of diurnal rhythms of migration does not argue against the nervous control, and neither do the effects of light. The rate of movement is admittedly slow compared with speed of muscular movements in insects, but the movement does not appear to be muscular in nature. Reasons have been given above for discounting the evidence concerning the apparent absence of a nerve supply to the pigment cells. The remote possibility of the liberation of the appropriate chemical mediator occurring as the result of antidromic impulses along the sensory nerves should not be overlooked.

The action of chloretone, carbon dioxide, light, and low temperature would be interpreted on the nervous theory as agents which cause cessation or decrease of impulses from the brain which normally maintain the pigment cells in the dark position. There is actually no positive evidence in favor of the nervous control since no method has been found for any arthropod which induces movement into the dark position, except darkness itself.

In addition, it has been shown in the above experiments that changes in the phototactic behavior of *Ephestia* are not entirely dependent upon the position of the accessory pigment cells. Nevertheless, the variation in the position of the pigment should be considered in investigations on the behavior such as that of Brandt (1934), and may explain some of the irregularities which he reported. Likewise, the results reported by Taylor and Nickerson (1940) on changes in retinal potentials during light adaptation should also be considered in relation to the pigment migration which doubtless occurs in *Galleria* as in *Ephestia.* The possibility of relating electrical response to migration of eye pigments has already been suggested in the case of some beetles by Jahn and Crescitelli (1940).

Summary

The accessory pigment cells in the eyes of *Ephestia kuehniella* migrate from their distal position between the cones to a more proximal position when exposed to light of sufficient intensity.

A comparable, or even more marked, effect is produced by low temperatures, chloretone, high tensions of carbon dioxide, and by cutting the optic tract.

Movement of individual cells can be induced by illuminating only a few of the ommatidia. This suggests that a hormonal method of control is unlikely. The fact that pigment cells can never be induced to migrate into the dark position once the optic tract has been severed suggests that the cells do not respond as independent effectors.

In view of these several lines of evidence, though most of it is admittedly negative, it is possible that the migration of the accessory pigment cells in the eyes of *Ephestia* may be principally controlled by a nervous mechanism. It should again be emphasized that any conclusions based on the study of moths cannot necessarily be applied to other insects, let alone to Crustacea.

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THE EFFECT OF TEMPERATURE ON THE RIGHTING OF ECHINODERMS

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The righting response of echinoderms has engaged the attention of investigators since Romanes and Preyer first studied it in the eighties of the last century, but the interest lay, in most cases, in analyzing the activities of the nervous system, as they manifested themselves in the reaction to a change in the position of the body. The representatives of the phylum usually employed were the sea-urchin (Echinoidea), the starfish or sea-star (Asteroidea), and the brittle-star (Ophiuroidea), none of which possesses statocysts so important in the righting of higher animals. The sea-urchin and the starfish turn over by the action of their tube-feet, while the brittle-star, which has no tube-feet, depends entirely on the muscular action of its arms.

Jennings (1907), in his extended report on the behavior of the starfish, gave considerable space to the righting response, but did not touch upon the time-element. Likewise, Hamilton (1922), in a paper devoted entirely to the topic of righting in the starfish, dwelt only on the mechanism of the process, but Fraenkel (1928) furnished some interesting figures on the time it took certain starfish to right themselves. An earlier paper containing time data is the one by Glaser (1907), who studied movements of brittle-stars. Working on the sand-dollar, Parker (1927) made some observations on the time relations of the various phases of righting. His paper also contains numerous references to the literature of body-righting in echinoderms. However, none of the authors mentioned attempted to relate righting-time to temperature, and in the chapter on the echinoderms in *Principles of Animal Behavior* by Maier and Schneirla (1935) there is no mention of a temperature factor in the discussion of righting. Barnes (1937), who lists a great number of biological processes for which temperature characteristics were obtained, has nothing on the subject of body-righting, although he gives several references on the effect of temperature on locomotion, of which, according to Hamilton (1922), the righting of the starfish is one phase.

Methods and Results

In the present investigation recently collected echinoderms were kept in aquaria, with the temperature of the water naturally varying from

18° to 26° C., usually higher in the afternoon than in the morning. The particular specimen to be observed was transferred to a very large Petri dish, filled with sea water whose temperature was regulated by the continual addition of chilled or warmed sea water and gentle stirring, thus maintaining the selected temperature within one-half a degree C. The animal was allowed to remain in the warmed or cooled water for at least 15 minutes, and, judging by its behavior, it acquired the temperature of the new medium during that period. The procedure followed was to lift the animal in the water and lay it in the upside-down position, as symmetrically as possible, on the bottom of the dish. The righting was observed both from above and from the side.

In general, no righting response could be elicited below 10° C. and above 30° C., and most of the observations were made at temperatures varying from 14° to 26° C. As a rule, 10 to 20 trials were made at one temperature level, then the water warmed or cooled, and another series of tests made at the new temperature level. In some cases the righting-times at half a dozen different temperatures were determined in succession, and the same animal observed again later in the day, or after an interval of several days. The righting-time was measured by means of a stop-watch, and in most cases the time required to turn through an angle of 90°, as well as the total turning-over time, was noted.

Sea-urchins

These animals usually turned over in 1½–2 minutes, with the greater portion of the period (71–95 per cent) needed for the first 90°. Sometimes, after turning through an angle of 130–150°, the animal would fall with a thud, apparently of its own weight. At low temperatures, however, sea-urchins often failed to complete the righting, remaining at an angle of 30–40° to the horizontal for a long time. On the other hand, at high temperatures the animals were likely to remain in the dorsal position, continuously executing translational or rotational movements (about a vertical axis). At 15° C. it took, on the average, 3½ minutes for a sea-urchin to turn over, and the best performance was at 24–26° C., when the mean total righting time was about 80 seconds, with the first half of the turn carried out in 66 seconds. The disparity between the fractions of the total righting time taken to turn through the first, as compared to the second 90° was greater at lower than at higher temperatures.

There was no evidence of fatigue on subjecting the sea-urchins to repeated righting. For example, mean figures in seconds for successive series of ten trials in different specimens were 77 and 75, 83 and 93, 83 and 79. Figure 1*a* shows the relation between the reciprocal of the absolute temperature of the water and the logarithm of the speed of righting (reciprocal of the righting time in seconds). Applying the Van't Hoff-Arrhenius equation to the data upon which this figure is based, a temperature characteristic of 19,000 calories was obtained.

Through the courtesy of Professor H. L. Clark, I was able to test the effect of temperature on the righting of three spiny urchins. They behaved in every way like the common sea-urchins, although they depended for their turning on the movements of the large spines, rather than those of tube-feet. Their performance was best at 26° C., when they turned over, on the average, in less than 70 seconds.

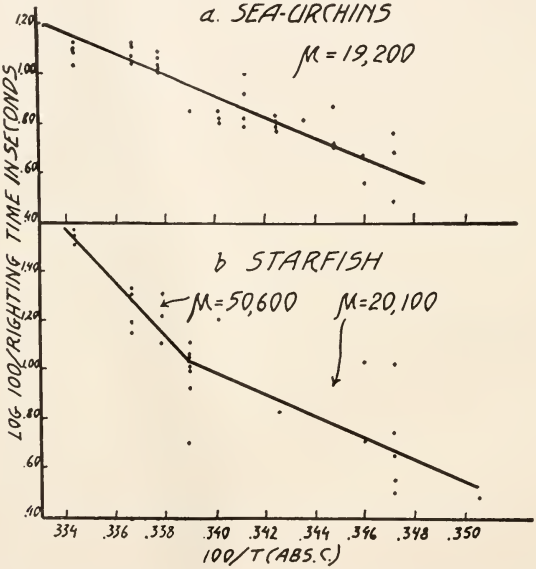

FIG. 1. The relation of the speed of righting, expressed as the logarithm of the reciprocal of the righting time in seconds, to the reciprocal of the absolute temperature of the water: *a,* for the sea-urchin, *Lytechinus variegatus atlanticus,* with one slope, and *b,* for the starfish, *Stolasterias tenuispina,* showing a break in slope at 22° C.

Starfish

As already stated, the characteristic features of the righting response of the starfish have been adequately described by Jennings, Hamilton, and others. Because several arms may participate in the turning process, sometimes interfering with each other, it is much harder than in the case of the sea-urchin to tell exactly when the starfish is half-turned. Therefore a certain position of the upper pole of the animal was accepted as indicating the midpoint of the response. Furthermore, as the arms often became entangled, the completion of the righting was also judged by the position of the upper pole, rather than the complete spread of the several arms.

At 6° C. the animals did not move at all, when in the upright position, and one arm was usually characteristically twisted and curled. Between 8° and 10° C. they would begin to stir, but did not crawl. Above 11° C. it was possible to obtain a righting response, first in 10–15 minutes, then in less time as the temperature was raised. As in the case of the sea-urchins, it took the starfish 66–93 per cent of the total righting time to execute the first half of the turn, and also as with the sea-urchins, the disparity between the fractions of time required to turn through the first and second 90° was greater at lower than at higher temperatures. The optimum temperature was 26° C., when the righting time was, on the average, 27 seconds. This figure compares well with the figures of Fraenkel (1928) for the righting time of " fast " starfish as 25 to 50 seconds, and of " slow " animals as one to three minutes.

Unlike the sea-urchins, the starfish showed evidence of some fatigue. One animal, kept at 21° C., gave the following figures, in seconds, for turning over, in two successive series of ten trials each: 41 to 60 and 54 to 122; another animal, at 23° C., showed a variation of from 55 to 89 seconds for the first ten trials, and 85 to 120 for the next. There was also a greater day-to-day fluctuation in the righting time of a particular starfish, at a certain temperature level, than there was in the sea-urchins. This resulted in a greater scatter of temperature-righting-time data, as plotted in Fig. 1*b*, but it is possible to discern two distinct trends in the curve, with a break at 22° C., and a temperature characteristic of about 20,000 cal. at lower temperatures and one of 50,000 cal. at higher.

Brittle-stars

These animals made no attempts to turn over at temperatures below 10° C., although the arms would execute undulating movements, and some could move short distances while in the dorsal position. At 14–15° C., they often required from 5 to 10 minutes to right themselves, thus not differing greatly from sea-urchins and starfish. At higher temperatures, however, they responded with great rapidity, their best time being only 5–6 seconds. On the other hand, brittle stars tired very quickly, first showing a marked lengthening of the righting time, then failing to respond altogether. For example, mean figures in seconds for successive series of ten trials on one animal at 22° C. were: 13, 22, 29, and in the fourth series only two responses of 27 and 53 were obtained, before the animal stopped responding to being placed on its back. Similar results were reported for brittle-stars by Glaser (1907), with the best righting times of only 3–4 seconds and rapid fatigue in some, though not in all animals tested.

The variability of the data as well as the fatigability of the animals resulted in such a scatter of the temperature-righting-time figures as to make it impossible to calculate a temperature characteristic of the process.

Discussion

The application of the Arrhenius equation to the relationship between the rate of biological processes and environmental temperatures has been confined mainly to such activities as enzyme action, oxygen consumption, carbon dioxide assimilation, embryological development, or to such organ and tissue performances as breathing, heart rate, nerve and muscle physiology. The only neuromuscular activities involving the organism as a whole to which this equation has been applied were locomotion, as cited by Barnes (1937), and reaction time of the human subject, as reported by Kleitman, Titelbaum and Feiveson (1938). In this investigation it was found that such a global process as body-righting is also subject to the effect of temperature. The echinoderms studied all failed to right themselves below 10–11° C., and at the lowest effective temperatures all took from 5 to 15 minutes to turn over. Although the righting time progressively decreased in all with a rise in temperature and the optimum performance attained in all at the level of 24–26° C., the shortest righting time was quite different for each of the three classes of echinoderms. It was longest (80 seconds) for the sea-urchin in which the process was least complicated, depending as it did on the action of successive groups of tube-feet, brought into play as the animal was turning over. It was shorter for the starfish (27 seconds), which, although it essentially depended on tube-feet action, had to follow the initiative of one or more of its arms, sometimes several arms working against each other in attempting to turn the animal in opposite directions. It was shortest (5–6 seconds) for the brittle-star that performed the righting by muscular action entirely.

On repeated testing the slowest of the three, the sea-urchin, showed practically no fatigue; the fastest, the brittle-star, tired very quickly; while the starfish, in this respect, too, occupied a middle position. Whether the ultimate failure to turn over was due to fatigue of the receptor or central nervous mechanism has not been established.

Concerning the temperature characteristics, it will be recalled that the righting of the sea-urchin had one μ value of 19,000, while that of the starfish had two: 20,000 below 22° C. and 50,000 above that temperature. Barnes (1937) states that when there is a break in the slope of the rectilinear relation between the logarithm of the rate of activity and the reciprocal of the absolute temperature, the μ values in the higher temperature range are usually smaller than those pertaining to lower temperatures. In the righting of the starfish the reverse was true, but there have been reports of many other biological processes with a greater μ value at higher temperatures, among them such a global activity as locomotion of the ant, studied by Barnes and Kohn (1932). Although it was impossible to obtain a definite μ value for the righting of the brittle-star, the available data suggest a high μ value at the upper temperature levels.

It may be added that, in taking 66–93 per cent of the total righting time to turn through the first 90°, the sea-urchins and starfish behaved like the sand-dollars studied by Parker (1927), who found that " the lift from the horizontal to the vertical requires as much as 3 hrs.; the drop from the vertical to the horizontal about half an hour." The similarity is particularly striking in that the sea-urchins and starfish turned in water and their total righting times were expressed in minutes or seconds, while the sand-dollars partly buried themselves in sand and took several hours to turn over.

Summary

The speed of a global activity of three echinoderms, in the form of body-righting, is related to their temperature, within the physiological limits of 10° to 30° C. The speed increases as the temperature rises, and optimum performance is obtained at 24–26° C.

ACKNOWLEDGMENTS

I wish to thank Professor H. L. Clark of the Harvard Museum of Comparative Zoölogy for advice and assistance, and for furnishing me with the names of the animals studied, which were as follows: common Bermudian sea-urchin—*Lythechinus variegatus atlanticus* (A. Agassiz); stout-spined sea-urchin—*Eucidaris tribuloides* (Lamarck); starfish or sea-star—*Stolasterias tenuispina* (Lamarck); and brittle-star—*Ophiocoma echinata* (Lamarck).

It is a pleasure to express my gratitude to Dr. J. F. G. Wheeler, the Director of the Station, who placed its facilities at my disposal.

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THE EFFECT OF PHYSOSTIGMINE ON THE RESPONSES OF EARTHWORM BODY WALL PREPARATIONS TO SUCCESSIVE STIMULI

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INTRODUCTION

The experiments reported in this paper were performed in an attempt to analyze certain augmentation phenomena in the responses of the body wall of the earthworm, *Lumbricus terrestris,* and to provide some basis for an opinion as to their cause.

One type of augmentation concerned in these investigations is that of summation of contraction. When a second stimulus follows the first before the response to the first is completed, the second contraction is superimposed upon the first to produce a greater muscular response. With an increase in the frequency and number of stimuli a condition of tetanus is produced.

Another type of augmentation is shown by the muscles of the body wall when a second stimulation follows the first after the response to the first is completed. In a series of stimulations each successive response is greater, resulting in a " staircase " effect.

The various augmentation phenomena which are shown by striated, smooth, and cardiac muscle of vertebrates have been studied extensively by many workers. Among the invertebrates, the field is unexplored except in coelenterates, echinoderms, mollusks, and crustaceans. Because of the diverse neuromuscular mechanisms involved in these different types of invertebrates, the augmentation phenomena themselves present varying characteristics, some being comparable to those found in vertebrates, while others are peculiar to a particular invertebrate group.

The first evidence for summation in the body wall muscles of the earthworm was given by Budington (1902), whose records showed an increase in response corresponding to an increase in the number of shocks administered. After Budington there is no other mention of augmentation phenomena in the earthworm until the studies of Bacq and Coppée (1937), who included three experiments on the earthworm in their work on *Sipunculus* and the leech. They found that physostigmine increased the muscular response when the nerve cord of the earthworm was stimulated repetitively.

The purpose of this paper is to show, first, how the muscular responses of the earthworm body wall are affected by variations in the frequency and number of single shocks and by the spacing and duration of tetanic stimulations; second, that the ability of the muscle to give augmented responses depends upon these time factors; and third, that the effect of physostigmine upon the muscle responses suggests the participation of acetylcholine as a facilitating factor.

An investigation of the muscular responses of the earthworm body wall is of especial interest since this muscle seems to parallel vertebrate striated muscle in some of its physiological characteristics. Certain of these similarities have been referred to by Pantin (1935*b*) and by Wu (1939).

MATERIALS AND METHODS

The specimens of *Lumbricus terrestris* used for these experiments were kept in an ice-box in moist earth and only the large and healthy specimens employed.

After partially anesthetizing the earthworm in 0.2 per cent chloretone, a mid-ventral slit was made the length of the worm and the nerve cord and digestive system were removed. This preparation of the body wall will be referred to as the muscle strip. At one end it was pinned to a paraffin block and at the other it was attached to a lever of spring steel, which recorded the contractions of the longitudinal muscles on a kymograph drum by a downward deflection of the lever. The approximate magnification of the lever was five times.

Another preparation used was the whole worm minus about the first ten segments, arranged for recording muscle contractions in the same way as described for the muscle strip. This will be referred to as the whole worm preparation.

For stimulating both the muscle strip and the whole worm, a fine silver wire electrode leading from a vacuum tube stimulator was inserted in each end of the preparation. The stimulator employed a gas triode 885 arranged to deliver stimuli at frequencies from 1 to 100 per second. The duration of the bursts of stimuli and the interval between the bursts were controlled by a commutator in the circuit. Submaximal stimuli were used in all the experiments described in this paper unless indicated otherwise.

RESULTS

The Response to Successive Single Shocks

When the body wall preparations were stimulated electrically by a series of submaximal single shocks, the type of response was found to be affected by the frequency of the shocks.

With a frequency of about 2 per second the second response showed an increased contraction, but after that there was no further augmentation. (See Fig. 1, *B*.) With an increase in frequency there was a successive increase in the magnitude of the first four contractions. A small amount of tonus developed which persisted for a short time after the stimulation stopped. (See Fig. 1, *A*.) Increasing the frequency to 6 per second caused an augmentation in the successive responses up to the ninth, which was nine times greater than the first response. This was followed by an increase in tonus and a diminution in the magnitude of the individual responses. (See Fig. 1, *C*.) The record shows a definite "staircase" effect. Strictly speaking, the term "staircase" should be reserved for the increased muscular responses brought about with maximal stimuli, thus showing that the heightened responses are due to increased contractions of the individual contractile units involved. Since maximal stimuli were not used in these experiments, the descriptive term of "augmentation of responses" is employed, and the determination of the exact mechanism of the facilitating effect is left for future investigation.

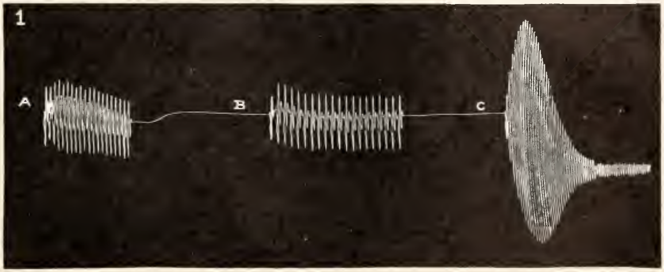


FIG. 1. Effect of frequency of single shocks on augmentation of contractions. Whole worm preparation. Frequency in shocks per second: *A*, 3 per second; *B*, 2 per second; *C*, 6 per second.

With a frequency of 10 per second a state of increased tonus is produced immediately because of the summation of the successive contractions, and with a frequency of about 14 per second, the response shows a condition of completely sustained contraction or tetanus.

Summation of the Responses to a Series of Shocks

This muscle preparation gives a response to a single shock and so can be called a single volley muscle. Records were made on a stationary drum of the responses of the whole worm preparation to one, two, and three single shocks delivered within a 0.3-second period. The response to two shocks was about one and one-half times greater than it was to one, and the response to three shocks was over twice as great.

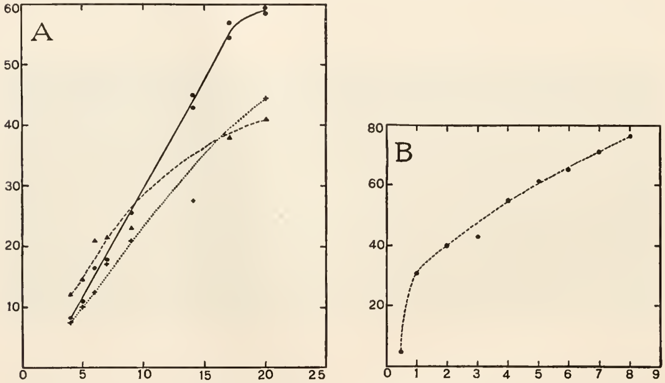


FIG. 2. *A*. Frequency-response curves for muscle strip. Abscissae, number of shocks. Ordinates, response in mm. Duration of bursts, 0.5 second. Interval between bursts, 1 minute. First series shown by dotted line. (Second series omitted.) Third series, unbroken line. Fourth series, broken line. There is a ¾ hr. interval between series 3 and 4, a 10-minute interval between the other series.

B. Duration-response curve for whole worm preparation. Abscissae, duration of burst. Ordinates, response in mm. Frequency, 14 shocks per second.

In order to show the effect of frequency upon the magnitude of contraction, a series of responses to a single burst of stimuli was recorded. The duration of every burst was constant, but the frequency of the stimuli within each burst was varied at random over a relatively wide range. A long interval was allowed between the bursts in order to prevent an effect of previous activity upon the response. The responses were recorded on a stationary drum and the length of each measured in millimeters. From the results obtained, frequency-response curves were made. Figure 2, *A* shows three of these curves for one muscle strip.

The magnitude of the responses increases with each increase in the number of shocks. The increase throughout the first series of trials is nearly in direct proportion to the number of shocks. In the third series the increase in responses is proportional to the number of shocks except with the highest frequency where there is a marked decline in the amount of augmentation. The fourth series was recorded three-quarters of an hour after the third series and about two hours from the beginning of the experiment. Although with the lower frequencies the muscle gives greater contractions than before, with an increase in the number of shocks the amount of augmentation declines, so that with the higher frequencies the responses are lower than in the other two series. This decline is probably due to the deterioration of the preparation.

These results show that the augmentation is proportionally less with the higher frequencies. There is an indication of a slow cumulative building-up process, since the responses of the second series were greater than the first, and those of the third the largest of all.

In one experiment with the whole worm preparation in which the duration of the bursts was gradually increased while the frequency was kept constant, the magnitude of the response to a burst was increasingly greater from the first response measuring 6 mm. up to the tenth measuring 76 mm. A duration-response curve was made by plotting the magnitude of the responses against the duration of each burst. (See Fig. 2, *B*). Experiments in which the duration of the bursts was changed at random showed the same effect of increased contractions due to longer bursts.

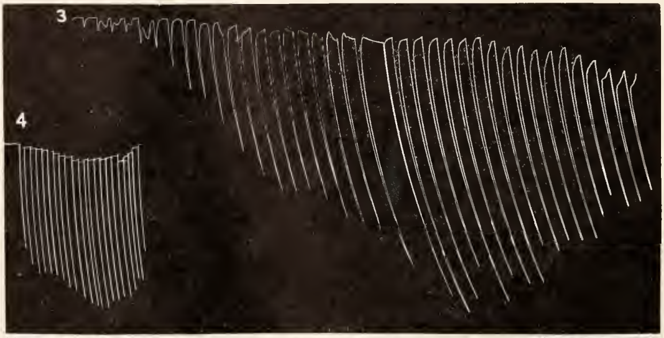
These results show that the magnitude of a response to one isolated burst of stimuli is affected by the frequency of the stimuli within the burst and the duration of the burst.

The Response to Repeated Bursts of Stimuli

When the earthworm body wall is stimulated electrically with shocks at a constant frequency delivered in repeated bursts at appropriate intervals, the first contractions show a definite increase in each successive response. When this augmentation ceases, it is not followed by a plateau, but by an immediate but gradual decline in the magnitude of the successive responses. The rate of decline varies under different conditions of stimulation.

Figure 3 shows the first part of a normal curve for the whole worm preparation. Since the frequency of stimuli within the bursts was 28 per second, these responses were tetanic in character. The first 22 records of contraction show an increase in the response to each successive burst. Figure 4 shows the same phenomenon in a muscle strip when the same frequency is used. A longer interval was allowed between bursts in the case of the muscle strip preparation because the muscle strip required a longer period to recover its original state of tension after each response.

To investigate the characteristics of the "staircase" effect exhibited by these muscles, many series of responses were recorded showing the effect upon the augmentation phenomenon of four easily variable conditions: the intensity of the stimulating current, the duration of the bursts of stimuli, the interval between the bursts, and the frequency of the stimuli within the bursts. It should be noted that in some instances it is impossible to make exact quantitative statements which apply to all



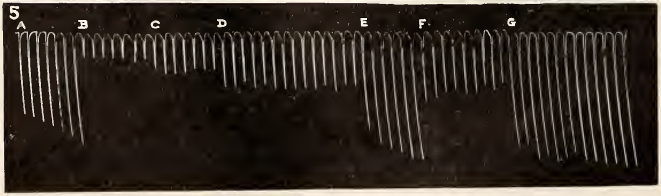


Fig. 3. Augmentation of contractions in whole worm preparation. Duration of burst, 0.3 second. Interval between bursts, 7 seconds. Frequency, 28 shocks per second.

Fig. 4. Augmentation of contractions in muscle strip. Duration of burst, 0.4 second. Interval between bursts, 27 seconds. Frequency, 28 shocks per second. Drum stationary.

Fig. 5. Effect of frequency of stimuli within burst on augmentation of contractions. Whole worm preparation. Duration of burst, 0.3 second. Interval between bursts, 7 seconds. Frequency of stimuli: *A, E, G,* 28 shocks per second causes augmentation; *B,* 9 shocks per second; *C,* 14 shocks per second; *D, F,* 18 shocks per second.

the preparations, since the differing physiological states of individual worms produced variation in response.

Difficulties were encountered in working with maximum intensities. In the case of muscle strips, a single burst of stimuli of high intensity induced a condition of tonus which was prolonged to such an extent that successive responses could not be elicited. With the whole worm preparations, high intensities often brought about strong spontaneous contractions which make it impossible to continue with the experiment. In the few successful experiments with high intensities there was no augmentation of the successive responses. This is a crucial point which should be investigated more thoroughly by further experimentation. Because of the disadvantages presented by the use of high intensities, submaximal stimuli of uniform intensity were used in the following experiments.

The length of the interval between bursts has a very definite effect upon the production of augmented responses. Using a frequency of 28 per second with the duration of burst of 0.3 second, there is a striking increase in successive responses in the whole worm preparation when the interval between bursts is 7 seconds. On doubling this interval, there is still some augmentation, but as the interval is increased still more this is less evident, until, with a 30-second interval, the successive responses show no increase.

The duration of the bursts also affects the production of augmented responses. In one experiment in which a frequency of 18 per second was used with bursts spaced at 14-second intervals, the responses were not facilitated when the bursts lasted 0.4 second. Lengthening the bursts to 1.5 seconds built up increasing contractions through six successive responses, the sixth one being more than twice as great as the first.

The frequency of the stimuli within the bursts is a third factor in determining the production of augmented responses. A low frequency does not bring about an increase in the successive responses. Figure 5 shows a long series of responses of the whole worm preparation to bursts of stimuli. The evident arrangement in groups is due to the different frequencies of the stimuli. A frequency of 18 per second or less did not cause augmentation, but with a frequency of 28 per second, three of the groups in the series show facilitated responses. In other preparations, however, increasing contractility was sometimes brought about with lower frequencies.

From the results of these experiments and many others, it is evident that the phenomenon of augmentation is to a great extent dependent on the number of stimuli applied in a unit of time and the grouping of these stimuli. As the interval between bursts is lengthened, either the frequency of the stimuli within the bursts must be increased or the duration of the bursts must be lengthened in order to produce a series of increasing responses.

The Effect of Physostigmine upon the Production of Augmented Responses

In order to determine the underlying cause of the facilitation phenomenon which is manifested in the augmented contractions of the earthworm body wall muscles, application of physostigmine was tried. Wu (1939) has shown that the sensitivity of the body wall to acetylcholine is greatly increased by physostigmine. From the results of his experiments concerning the action of drugs on the earthworm body wall, he postulated the presence of some factor which prevented the action of

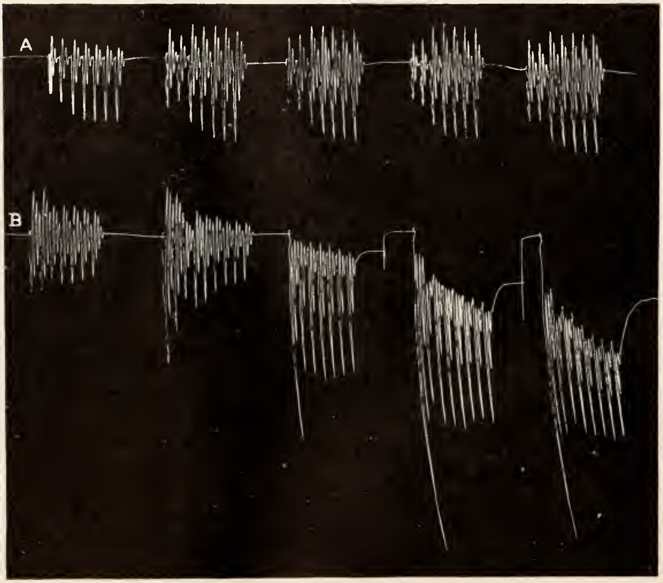


FIG. 6. Effect of physostigmine 1 gm./10,000 cc. on the response of whole worm preparation to a series of single shocks. Ten successive series of shocks each consisting of 7 single shocks in 5 seconds separated from one another by an interval of 3 minutes. Series *A*, Ringer's drip. Series *B*, physostigmine drip.

acetylcholine and which was antagonized by physostigmine. This he thought was probably a high concentration of choline esterase.

If acetylcholine is produced by stimulation of the earthworm muscle preparation, and if this is not completely hydrolyzed by choline esterase before the next stimulation, the persisting acetylcholine could be the cause of the augmentation of the responses. The application of physostigmine which prevents the action of choline esterase could, therefore, show some effect upon the augmentation phenomenon. The following experiments were devised to test this hypothesis.

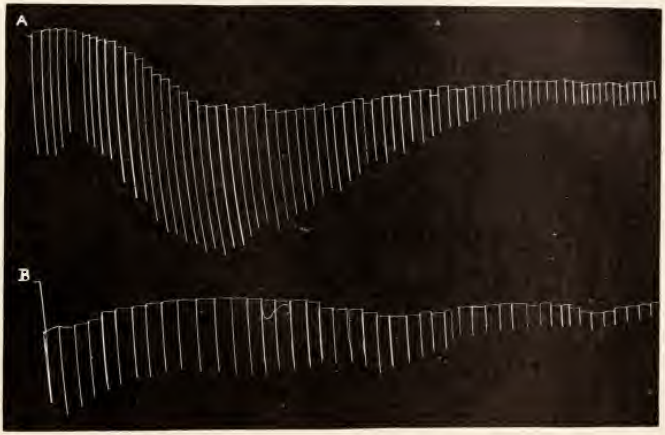


FIG. 7. Effect of physostigmine, 1 gm./10,000 cc., on the responses of muscle strip to a series of bursts of stimuli. Interval between bursts, 27 seconds. Frequency, 40 shocks per second. Drum stationary. *A*, physostigmine applied by drip method during experiment. *B*, muscle strip immersed in physostigmine for 15 minutes previous to stimulation.

The effect of physostigmine on the responses to a series of single shocks was to produce a striking augmentation. The whole worm preparation was stimulated for 5 seconds at a low frequency of 7 shocks in 5 seconds. This was followed by a 3-minute period of rest during which the preparation was given Ringer's solution by drip method. Figure 6, *A* shows a series of five of these 5-second stimulations. Within each of the five groups there is an increase in the successive responses, but the facilitation decays during the 3-minute rest period, so that the first response in each of the five groups is of the same magnitude. Figure 6, *B* shows the result of repeating this procedure except that physostigmine drip 1 gm./10,000 cc. was substituted for the Ringer's

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during the 3-minute rest period. Here the first response in each group is increasingly greater. The succeeding responses in one group are smaller than the first response of that group but greater than the normal responses shown in Fig. 6, *A*. A state of maintained tension develops during each 5-second burst of stimuli. The optimum action of the physostigmine, judged by magnitude of response, was reached in 12¼ minutes, as shown in the fourth series, in which the maximum response is 8½ times greater than the first response in the normal preparation. The fifth series shows no further increase in magnitude of response.

In order to show the effect of physostigmine upon a series of successive tetanic responses, physostigmine 1 gm./10,000 cc. was applied to a muscle strip preparation continuously by the drip method during a long series of bursts of stimuli. Figure 7, *A* shows the result. A strong tonus was built up gradually in the first 18 responses, but even with this increasing tonus there was an augmentation of the successive individual contractions. On the other hand, when the preparation was put into a bath of physostigmine for 15 minutes before the stimulations began, the first response was of normal magnitude, but following this response an immediate tonus was manifested. The second response was only three-fourths the magnitude of the initial one, and the succeeding responses showed a fatigue-like diminution. The long application of physostigmine prevented any augmentation of contractions. These characteristics can be seen in Fig. 7, *B*. Several control experiments, in which the preparation was left in a bath of Ringer's solution for 15 minutes previous to stimulation, showed a normal response.

TABLE I

Effect of physostigmine on the magnitude of the responses. Two muscle strips were used: *A*, with physostigmine drip 1 gm./10,000 cc.; *B*, with Ringer's drip. Duration of bursts, 0.3 second. Interval between bursts, 3 minutes. Frequency, 40 shocks per second. The magnitude of response is recorded in the body of the table in mm.

Successive responses	1	2	3	4	5	6	7	8
A. Physostigmine	31	38	45	50	51	53	52	55
B. Ringer's	40	43	43	43	40	40	43	40

Physostigmine was demonstrated to increase the length of the interval between bursts of stimuli which is necessary for the production of augmented responses. As has been stated above, there is a maximum interval of about 30 seconds, which, if exceeded, does not allow augmentation. When a normal muscle preparation is stimulated by bursts which are separated by 3-minute intervals, there is no increase in the successive responses. Table I shows the magnitude of the responses as measured by the length of the record in millimeters for two muscle strips, one treated with physostigmine drip 1 gm./10,000 cc., the other with Ringer's. When physostigmine drip was used on the preparation, there was an increase in the magnitude of 8 successive responses even though these were separated by 3-minute intervals.

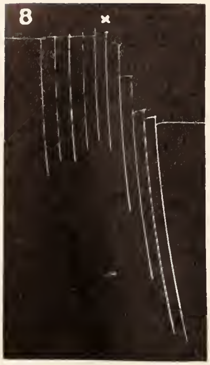
Figure 8 demonstrates this effect in a single muscle strip stimulated in the same way. The first five responses are separated by 3-minute intervals of treatment with Ringer's drip. There is no increase in the successive responses. After the fifth response, treatment with physostigmine drip, 1 gm./10,000 cc., is begun during the 3-minute interval with the result that augmentation is produced as well as an increase in tonus.

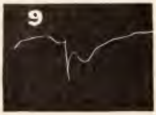
The experiments with physostigmine described above suggest that at each burst of stimuli acetylcholine was formed and that the physostigmine acted upon the choline esterase to delay the breakdown of this acetylcholine. This resulted in the persistence of a certain quantity of acetylcholine which caused an increase in the next response of the muscle.

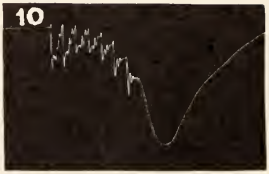
It will be noted that a relatively high concentration of physostigmine was used in these experiments. This was in order to favor the diffusion of sufficient drug into the tissues to produce an immediate effect even with the slow drip method employed.

Further indication of the formation of some facilitating substance at the time of stimulation is shown by conditions of tonus succeeding the muscular responses. Figure 9 shows a response in which the primary contraction and partial relaxation due to the single shock is followed by a smaller and slower contraction and relaxation. In Figure 10 is recorded the response to a series of 7 single shocks after the preparation had been in physostigmine 1 gm./100,000 cc. for 4 minutes. After the seventh response, the stimulation having stopped, there is a long slow contraction followed by relaxation.

The persistence of a facilitating effect is demonstrated in Fig. 11. Here a series of augmented responses was produced using a frequency of 28 per second. This was followed, with no break in the intervals of stimulation, by a series of bursts with a frequency of 9 per second, which is not a facilitating frequency. The first four responses to the low frequency bursts show a greater response than those which follow. The preceding series of augmented responses had a facilitating effect which lasted 29 seconds. This corresponds to the interval of 30 seconds determined to be the maximum interval within which facilitation can occur. The response to a single shock is also increased when immediately preceded by a series of augmented responses.







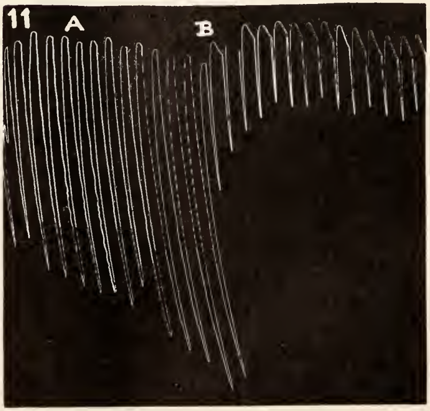


FIG. 8. Effect of physostigmine 1 gm./10,000 cc. in producing augmentation of contraction in a muscle strip with an interval of 3 minutes between bursts of stimuli. *X* represents physostigmine drip begun. Duration of bursts, 0.3 second. Frequency, 40 shocks per second.

FIG. 9. Response of muscle strip to a single shock followed by a change in tonus.

FIG. 10. Responses of muscle strip to a series of 7 single shocks after 4 minutes in physostigmine 1 gm./100,000 cc. Following the responses there is a change in tonus.

FIG. 11. Increased magnitude of the initial responses to bursts of low frequency when preceded by a series of successively augmented responses. Whole worm preparation. Frequency of stimuli: *A*, 28 shocks per second; *B*, 9 shocks per second. Duration of burst, 0.3 second. Interval between bursts, 7 seconds.

Discussion

From the results of the experiments described it is evident that previous activity has a definite facilitating effect upon subsequent contractions of the longitudinal muscles in the body wall of the earthworm.

When preparations are stimulated with successive single shocks, three types of responses are possible, depending upon the frequency of the shocks: first, an increase in magnitude of successive separate contractions; second, a partial summation of the successive contractions; and third, complete tetanus. Since, with increase in frequency of shocks, the first type of response merges gradually into the second, and the second into the third, it would appear that the same facilitating factor is responsible for all. This is also indicated by the fact that with the application of physostigmine it is possible to produce a summation of contractions or tetanus with the lower frequencies.

In his work on Actinozoa, Pantin (1935*a*) found that in different neuromuscular mechanisms he was able to demonstrate these three types of responses depending upon the time relation between the duration of a complete contraction and relaxation of the muscle, and the duration of the persistence of the facilitating factor.

In the earthworm preparations the facilitating factor persists between 0.4 and 0.5 second after a single shock. With repeated shocks the facilitation effect is cumulative, resulting in considerably heightened contractions, as exhibited by the responses to repeated tetanic stimulations. Under these conditions the facilitating factor may last nearly 30 seconds, as shown by an increased response to a second tetanic stimulation within that period of time.

This phenomenon of facilitation has been demonstrated in the muscular reactions of other invertebrates. The number and frequency of stimuli are the decisive factors in producing the augmented responses, as is the case in the earthworm, but the cause of the facilitation seems to be different in the various neuromuscular mechanisms.

Pantin (1935*a*), in his work on facilitation in Actinozoa, came to the conclusion that in certain muscles the increasing magnitude of the responses in a series of stimuli is due to neuromuscular facilitation, by which, with each succeeding stimulus more muscle fibers are affected. He found no evidence at that time for the functioning of chemical mediators in coelenterates. Ross and Pantin (1940), in their investigation of the effect of certain ions on facilitation in Actinozoa, found that two factors were involved in facilitation. Although they did not determine the nature of the facilitating process they concluded that it could not be due solely to a transmitter.

In crustacean striated muscle, according to Katz (1936), the frequency to which individual muscle fibers respond varies, so that the number of fibers which contract and consequently the magnitude of the contraction, are controlled by the frequency of the nerve impulses.

In the present paper evidence has been offered to support the view that in the earthworm body wall the facilitating factor is acetylcholine. The effect of physostigmine in producing greatly augmented responses points to this. The most convincing evidence is the ability of physostigmine to delay the decay of the facilitating factor, so that augmented responses are elicited even with long intervals between tetanic stimulations.

The location of this facilitating effect is a subject for further investigation. Since the phenomenon showed the same characteristics in the muscle strip as in the whole worm preparation, it cannot be dependent on the nerve cord.

SUMMARY

1. The longitudinal muscles of the body wall of the earthworm (*Lumbricus terrestris*) show augmented responses when stimulated by successive single shocks at low frequencies. The facilitating condition lasts not more than 0.5 second after the response to a single shock.

2. A frequency of 14 per second results in a complete summation of the contractions or a condition of tetanus.

3. The magnitude of a summated response elicited by a series of shocks is proportional to the duration of the burst of shocks and to the frequency of the shocks within the burst.

4. The tetanic responses to repeated bursts of stimuli show an increasing augmentation of the initial contractions. The production of this "staircase" effect is affected by the frequency of the shocks, the duration of the bursts, and the length of the interval between bursts.

5. After a brief tetanus the facilitating condition persists for nearly 30 seconds.

6. The application of physostigmine increases the augmentation of responses and tends to produce a condition of tonus.

7. Physostigmine delays the decay of the facilitating property so that augmented responses are produced with intervals as long as 3 minutes between bursts of stimuli, suggesting the rôle of acetylcholine in the production of the augmented responses.

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THE RELATION BETWEEN THE FOUR-CARBON ACIDS AND THE GROWTH OF OAT SEEDLINGS

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INTRODUCTION

Plant growth hormones are noted for the multiplicity of their effects. This is particularly true of the auxins, which are known to influence elongation of roots and shoots, determination of root number, production of callus tissue, tropistic responses, correlative growth of plant organs and protoplasmic streaming.

The most striking feature about the variety of these auxin effects is the fact that any one concentration of the hormone will often stimulate one process, inhibit another, and have no effect on a third. If the concentration is varied these relations may be completely altered. Such effects, together with the fact that the auxins have an effect on protoplasmic streaming, have suggested that these hormones must play a decisive rôle in some fundamental and common intracellular process.

For some time attempts had been made to demonstrate a link between auxin and cell respiration. Experiments showed, however, that auxin had no demonstrable effect on the respiratory rate of plant cells (Bonner, 1936; Van Hulssen, 1936) and the conclusion was drawn that the hormone had no respiratory effect (DuBuy, 1940).

More recently, however, Commoner and Thimann (1941) have shown that indole-3-acetic acid is directly related to the activity of a specific respiratory process, the 4-carbon dicarboxylic acid system. They showed that the salts of these acids (malic, fumaric, and succinic) will enhance the effect of auxin on growth and that the inhibitory effect of iodoacetic acid on growth is due to a specific inhibition of this system. It was shown that the 4-carbon acid system normally accounted for only 5–10 per cent of the total respiration, but that all the growth is dependent upon its activity. Thus it was demonstrated that auxin can stimulate the respiratory activity of this system (and in this way increase the rate of oxygen consumption) and that the effect of the 4-carbon acids on respiration is enhanced by the presence of auxin.

This work dealt with a single effect of auxin, the elongation of excised sections of the *Avena* coleoptile, but indicated that the effect of the hormone is exerted through a common cellular oxidative process.

The work suggested that this effect may play an important rôle in determining the nature and direction of the influence of auxin on the other processes mentioned above. This indication was also supported by the findings of Sweeney (unpublished data) that the 4-carbon acids influence significantly the auxin effect on protoplasmic streaming in the *Avena* coleoptile.

It was our purpose therefore to extend this evidence by examining the effect of one of the 4-carbon acids on three different growth processes known to be influenced by auxin in the intact *Avena* seedling: shoot elongation, root elongation, and determination of root number.

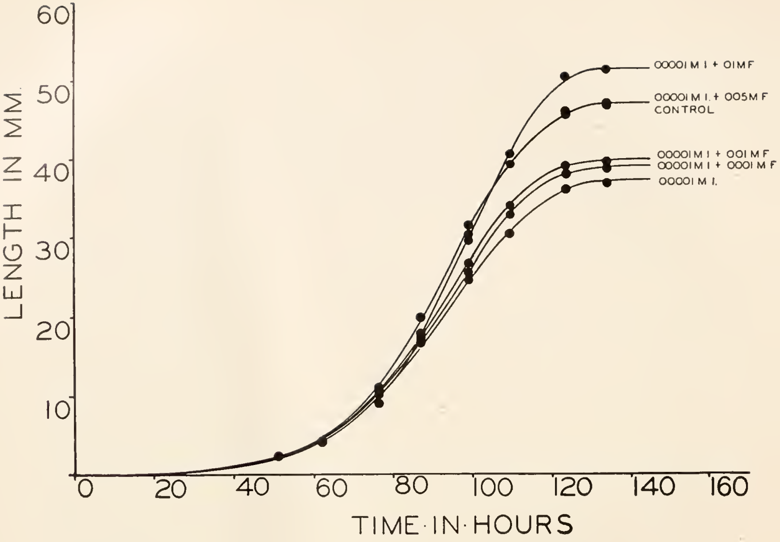
PROCEDURE

Seeds of *Avena sativa* L. var. Black Norway and Fulghum similar to those used in earlier experiments were hulled and soaked in distilled water for 20 hours at room temperature with continuous aeration. After soaking, the oats were placed in beakers lined with moist filter paper, as described in an earlier paper (Kaiser and Albaum, 1939), and to which the test solutions had been added. The plants were then allowed to continue their growth in the dark at room temperature. At various times the coleoptiles were measured to the nearest millimeter under an orange safelight. Growth of the first internode appeared to be completely inhibited; no effort was made to measure this separately. At the close of each experiment (i.e., when the coleoptile had ceased growing), final measurements were taken on total root length, total root number and final coleoptile length. All figures reported here are the averages of at least twenty plants. The test solutions, indole-3-acetic acid (Merck), iodoacetic acid (Eastman Kodak) and fumaric acid (Eastman Kodak) were made up in distilled water and adjusted to pH 6.0 with KOH at the beginning of each experiment. No change in pH occurred during the course of the experiment. Measurements on the length of epidermal cells were carried out at the close of the experiments by stripping the epidermis from small pieces of coleoptile, mounting it in water on a slide and measuring it with an ocular micrometer under the low powers of a compound microscope.

RESULTS

The Effect of Iodoacetate on Coleoptile Growth

These experiments indicate that the 4-carbon acid system influences the effect of auxin on seedling growth in a manner similar to its effect on the growth of isolated coleoptile sections.



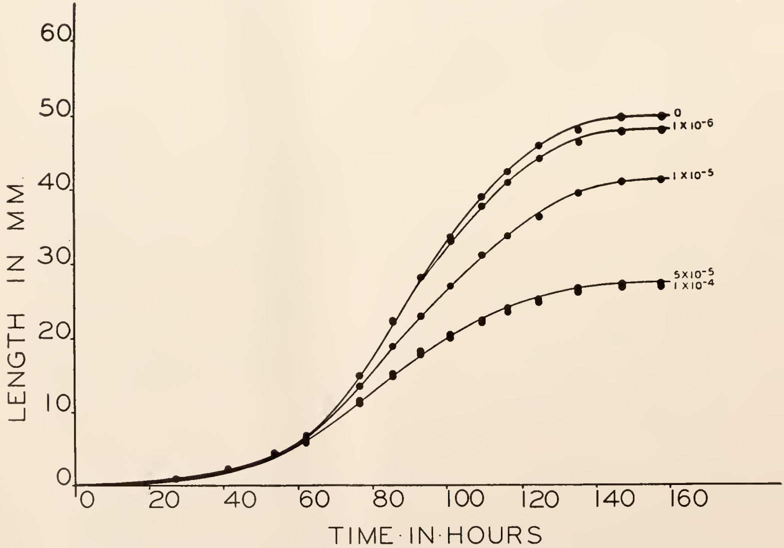


FIG. 1. Growth curves of coleoptiles following poisoning with iodoacetate, and recovery after addition of various concentrations of fumarate.

FIG. 2. Growth curves of coleoptiles after poisoning with various concentrations of iodoacetate.

Figure 1 shows that 10^{-5} M iodoacetate reduces the final size of the coleoptile, although the growth curve characteristics are not affected (the half-times of all curves in Fig. 1 are identical). If fumarate is added the inhibitory effect of iodoacetate is negated; in fact, use of a concentration of .01 M fumarate results in a coleoptile even exceeding in size that of the control.

Figure 2 demonstrates the effect of various concentrations of iodoacetate on coleoptile growth. Maximum inhibiton (50 per cent) is obtained at a concentration of 5×10^{-5} M, identical with the maximum inhibiting concentration found by Commoner and Thimann (1941).

Thus it is indicated that iodoacetate influences the growth of the coleoptile *in vivo* in the same way that it affects the growth of isolated sections. Very low concentrations of this substance produce a sharp decline in the rate of coleoptile growth and this inhibition may be completely negated by the presence of sufficient fumarate.[1]

TABLE I

The relationship between iodoacetate concentration, final coleoptile length and epidermal cell size.

Iodoacetate Concentration	Coleoptile Length (mm.)	Epidermal Cell Size (ocular units)
0	50.0	26.0
10^{-6} M	48.1	22.6
10^{-5} M	41.2	17.4
5×10^{-5} M	27.3	12.6
10^{-4} M	26.9	11.3

That this effect is exerted on the growth processes within each cell is shown by the data of Table I. These data indicate a fairly close proportionality between the effect of various concentrations of iodoacetate on the length of the entire coleoptile and the average length of the epidermal cells of these coleoptiles. Thus it is suggested that the effect of iodoacetate and fumarate is on some general auxin-sensitive process in the cell.

The Influence of Iodoacetate and Fumarate on Auxin-sensitive Processes

A further investigation of this hypothesis was made possible by the fact that auxin has several different effects on various parts of the growing plant. If the above effects are due to a direct influence on the activity of the auxin processes, then it should be possible to demonstrate an augmentation of all auxin effects by fumarate and an inhibition of these effects by iodoacetate (in the proper concentration). The following experiments bear this out.

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[1] Commoner and Thimann (1941) were able to get complete inhibition with 5×10^{-5} M iodoacetate. In these experiments, we were able to get growth inhibitions of only 50 per cent maximally. We believe that this difference is due to penetration phenomena which would be different for the intact seedling as compared to the sections.

Three auxin-sensitive growth processes were selected for comparison: coleoptile (shoot) length, total root length, root number. It is already well known that in certain concentrations auxin tends to increase the coleoptile length, decrease the root length and increase the number of roots. The influence of auxin, fumarate, iodoacetate, and various mixtures of these substances was determined in the usual manner. The data obtained are presented in Fig. 3.

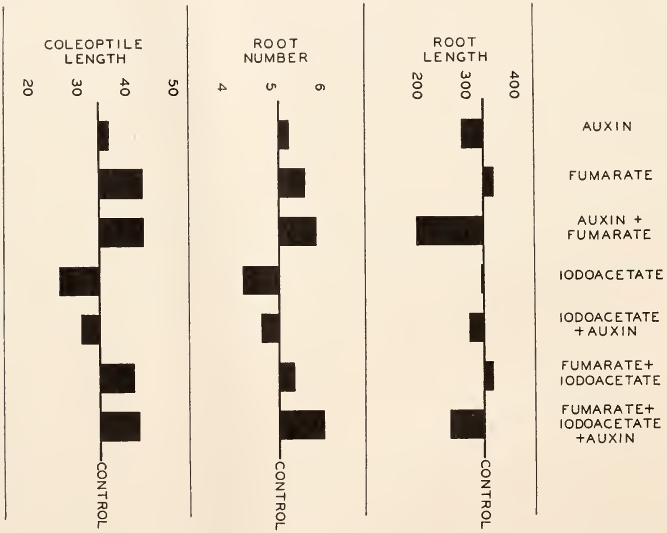


Fig. 3. Effects of auxin (10 mg./1.), fumarate (.01 M), auxin plus fumarate, iodoacetate (.00001 M), iodoacetate plus auxin, fumarate plus iodoacetate, fumarate plus iodoacetate plus auxin on the coleoptile length, root length and root number of *Avena* seedlings var. Black Norway.

This figure shows that, whatever the direction of the auxin effect (i.e., whether inhibitory or stimulatory), the effect is augmented by the addition of fumarate and inhibited by the addition of iodoacetate.

Thus, auxin reduces total root length; auxin and fumarate produce an even greater inhibition, while auxin and iodoacetate show a smaller inhibitory effect. Iodoacetate alone has no significant effect on root length, but reduces the number of roots and the coleoptile length. Auxin reduces this inhibitory influence of iodoacetate, in the case of the latter effects, but increases it in the case of root length. Fumarate alone acts like auxin in increasing coleoptile length and root number, but also has a slight positive effect on root length. In every case fumarate antagonizes the effect of iodoacetate.

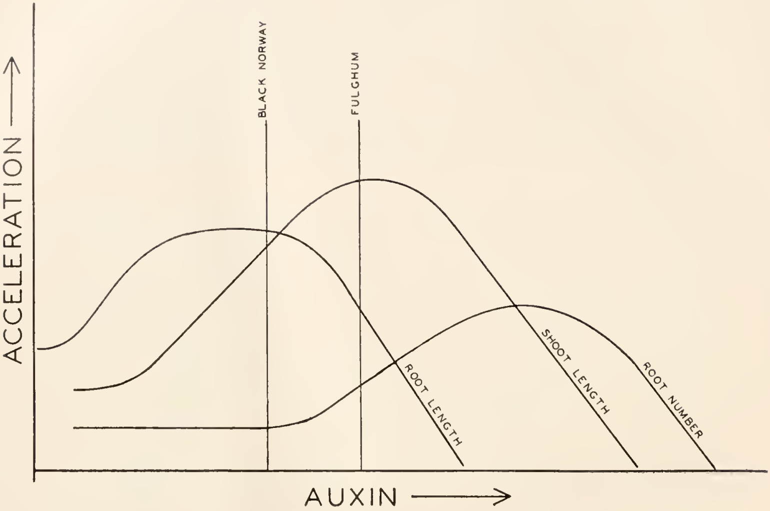
Thus it seems clear that the effects of iodoacetate and fumarate are exerted directly on these auxin-sensitive processes in the cells. The influence of these substances on the various growth relations in the oat seedling is directly related to the effect of auxin on these relations. It seems likely that the four carbon acids and auxin are together concerned with the activation of these processes, while iodoacetate is a specific inhibitor (in the proper concentration) of these processes.

The Differential Effect of Growth Substances on the Various Growth Processes

It has been suggested by Thimann (1937) that the effect of auxin on the various growth processes is essentially identical, but that the sensitivities of these processes to varying concentrations of auxin are different. He points out that the curve relating intensity of effect to auxin concentration is essentially the same for coleoptile length, root length, and root number, but that the zero points are different in each case. The suggested relation between these curves is shown in Fig. 4, together with the zero points indicated by the work of Kaiser and Albaum (1939) on two varieties of *Avena sativa,* Black Norway and Fulghum. The latter work showed that the variety Fulghum responded to auxin in a manner that indicated a greater intrinsic content of growth hormone as compared with Black Norway. The data on the effect of auxin in the table appended to Fig. 4 (taken from Kaiser and Albaum, 1939) indicate the probable validity of Thimann's suggestion. However, it is clear that the curves to the left of the zero lines were purely hypothetical since there was no method of quantitatively removing auxin from the plant.

It is apparent, from the data presented above, that iodoacetate does offer the possibility of investigation in the " negative " regions of these curves. Consequently, the effect of various concentrations of iodoacetate on the growth processes of the two varieties was determined. The data are shown in the table of Fig. 4.

It is clear that the response of these growth processes to iodoacetate follows the course of the curves to the left of the zero lines.



	IODOACETATE	0	$1X10^{-6}$	$1X10^{-5}$	$3X10^{-5}$	$5X10^{-5}$	$8X10^{-5}$	$1X10^{-4}$
BLACK NORWAY	SHOOT LENGTH	31.5	300	25.1	199	195	201	198
	ROOT LENGTH	324	312	320	244	220	218	224
	ROOT NUMBER	3.9	40	39	40	40	40	39
FULGHUM	SHOOT LENGTH	428	405	304	258	231	208	205
	ROOT LENGTH	256	278	285	282	265	264	214
	ROOT NUMBER	43	33	3.1	33	30	32	30
	AUXIN MG/L.	0	.005	01	02	05	5.0	50.0
BLACK NORWAY	SHOOT LENGTH	290	340		38.9			
	ROOT LENGTH	320		208		110	80	
	ROOT NUMBER	40				48	53	83
FULGHUM	SHOOT LENGTH	441	440		451			
	ROOT LENGTH	250		200		100	30	
	ROOT NUMBER	52				57	62	100

Fig. 4. Hypothetical relations between auxin concentration and its effect on root length, shoot length and root number (after Thimann, 1937). The vertical lines for Black Norway and Fulghum represent zero points or intrinsic hormone content as postulated by Kaiser and Albaum (1939). The auxin data in the table below are taken from the latter paper.

Thus, examination of the curves to the left of the Black Norway zero line indicates that progressive "removal" of auxin should show the following effects: on shoot length: an immediate decrease; on root length: no effect, followed by a decrease; on root number: no effect.

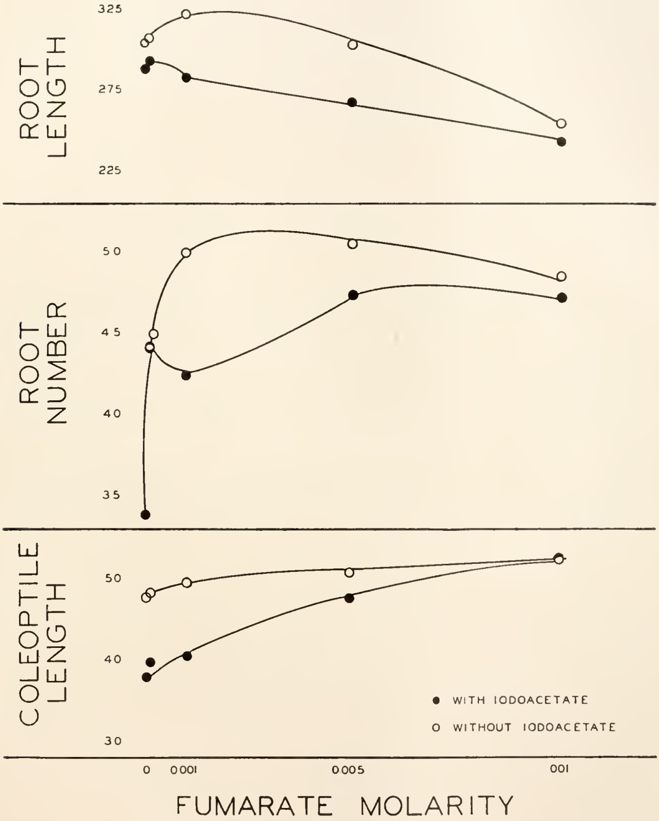


Fig. 5. Relationship between coleoptile length, root number and root length and the concentration of fumarate with and without .00001 M iodoacetate.

The data obtained follow this course. The shoot length is reduced by the lowest concentration of iodoacetate, a maximum inhibition being reached at a concentration of 5 × 10^{-5} M. Low concentrations of iodoacetate (up to 10^{-5} M) have no effect on root length, but larger amounts are inhibitory. Finally, all concentrations of iodoacetate used produce no significant change in the root number.

In the case of the variety Fulghum, the curves are also descriptive of the effect of increasing iodacetate concentrations. Since this variety contains a greater intrinsic concentration of auxin, the zero point is farther to the right. Thus, in this case, low concentrations of iodoacetate *increase* the root length, while higher concentrations *reduce* it. Similarly, low concentrations of the poison cause a slight reduction in root number, higher concentrations having no additional effect. There is an immediate inhibition of shoot length, again following the course of the curve.

It seems apparent that iodoacetate affects the growth processes in *Avena* by quantitatively inactivating the auxin originally present in the plants.

Such an effect might be taken to indicate a stoichiometric reaction between auxin and iodoacetate, resulting in inactivation of the hormone. However, such an explanation is contraverted by the marked *acceleration* of the growth of coleoptile sections by a concentration of 10^{-6} M iodoacetate (see Commoner and Thimann, 1941).

Furthermore, reversal of the iodoacetate inhibition by fumarate also seems to rule out this suggestion.

This is clearly shown by the data presented in Fig. 5. These curves illustrate the negation of iodoacetate inhibition by fumarate and show that there is no clearly proportional relation between the effects of these substances.

CONCLUSIONS

It is clear that the 4-carbon acid system is an important factor in the activity of auxin in controlling plant growth. There seems to be a close interaction between these factors, indicating that both participate in the various auxin-sensitive processes that regulate plant growth.

It is not our purpose at the present time to offer a complete explanation of this phenomenon, but the data presented demonstrate that the 4-carbon acids participate directly in the growth processes in the plant. The conclusions reached by Commoner and Thimann can therefore be extended to include many of the known effects of auxin.

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STUDIES IN SUBLITTORAL ECOLOGY

III. LAMINARIA FOREST ON THE WEST COAST OF SCOTLAND; A STUDY OF ZONATION IN RELATION TO WAVE ACTION AND ILLUMINATION

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INTRODUCTION

Rocky areas of the sea bottom in the shallow sublittoral region of the coast of Britain are in general densely covered with a forest of brown laminarian seaweeds. In this paper an account is given of the influence of depth on the distribution of organisms within the *Laminaria* forest, and of the factors which mainly control this distribution. Observations were made by means of a diving helmet (see Kitching, Macan, and Gilson, 1934), from low water of spring tides to a depth of about 12 meters below this level.

Carsaig Island, the place chosen for this work, is about 1 km. long and ⅕ km. wide, and lies in the Sound of Jura, on the west coast of Scotland (see inset to Fig. 1). The waters of the Sound of Jura in general reach a depth of 100–200 meters, and the tidal currents attain a velocity of 7–8 km. per hour; in windy weather there is a short choppy sea. According to measurements made in open water during hot still weather, the gradient of temperature within the shallow sublittoral region is insignificant; and it is probable that gradients of salinity, oxygen content, carbon dioxide content, and hydrogen ion concentration must also be slight. However, in places sheltered from wave action such gradients may well be set up. The western shore of Carsaig Island is fully exposed to wave action, but the eastern side is well protected. As will be shown below, the main features of the distribution of organisms within the shallow sublittoral region may reasonably be attributed to gradients of wave action, with all its consequences, and to illumination.

DISTRIBUTION IN THE LAMINARIA FOREST

Introduction and Methods

The *Laminaria* forest was investigated by observation from a boat with the help of a water telescope all around the coast of Carsaig Island, and by diving at several selected stations. Finally a detailed study was made, by diving, at one station (*A* on map, Fig. 1), down to a depth of about twelve meters below low water of spring tides. At this last station data were collected for the drawing of a diagrammatic section (Fig. 1) to show the vertical distribution of algae. Investigations were carried on in September of 1932, and in August of 1933–1936 inclusive. The general description which follows is based on all these observations.

Dominant Large Brown Algae

The upper margin of the sublittoral region,—that part which lies just above low water of spring tides,—is very different in character from the deeper layers. It is dominated by three large brown algae, *Himanthalia lorea, Alaria esculenta,* and *Laminaria digitata* (see Fig. 1). *Alaria* is confined to the west side of the island, and favors vertical rock surfaces as well as wave action. *L. digitata* and *Himanthalia* range all around the island. All three grow sufficiently densely to protect the organisms amongst them to a large extent from desiccation, so that the associated fauna is typically sublittoral.

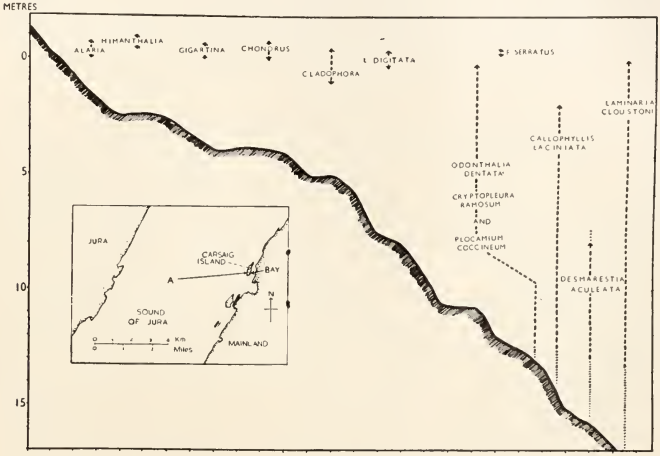
METRES
0
5
10
15
HIMANTHALIA
ALARIA
GIGARTINA
CHONDRUS
CLADOPHORA
L DIGITATA
F SERRATUS
LAMINARIA CLOUSTONI
CALLOPHYLLIS LACINIATA
ODONTHALIA DENTATA
CRYPTOPLEURA RAMOSUM
AND
PLOCAMIUM COCCINEUM
DESMARESTIA ACULEATA
JURA
CARSAIG ISLAND
BAY
A
N
SOUND OF JURA
0 1 2 3 4 Km
0 1 2 Miles
MAINLAND

Fig. 1. Section at position *A* (see inset) on coast of Carsaig Island. Inset: map showing position of Carsaig Island in Sound of Jura, Argyll, Scotland.

Just below low water of spring tides lies the lower limit of *Laminaria digitata,* and, except in extreme shelter, the upper limit of *Laminaria Cloustoni.* There is very little mixing of the two species. *Laminaria Cloustoni* in its zone forms a dense forest, but does not reach its greatest size at depths less than 4 meters. Here the innumerable vertical stipes support an immense canopy of fronds, below which the light is dim even when the sun is shining brightly above. In order to penetrate this forest without risk of the air line becoming entangled, we had to cut a glade with shears. The forest reaches a height of about 3 meters above the bottom. Individual plants of *L. Cloustoni* reach a length of 2 meters or more, the longest measured being 2.40 meters; and scattered plants of *Saccorhiza bulbosa* reach about the same length. The full height of the forest is made up by epiphytic *Laminaria* plants growing on the stipes of *L. Cloustoni.* These epiphytic plants are chiefly *L. digitata,* which, however, is rarely if ever found growing directly on the sea bottom below its normal zone. In general *Laminaria* spp. from deeper down or from the sheltered side of the island were found to have undivided or incompletely divided fronds, in contrast to the plants growing nearer the surface and on the wave-exposed side. Presumably wave action is most vigorous near the surface of the water.

TABLE I

Population of Laminaria digitata from the sublittoral fringe

	Individuals per 5 square meters of frond	Individuals per 10 stipes	Individuals per 10 holdfasts
On east (sheltered) side of Carsaig Island:			
Patina pellucida	31	0	1
Spirorbis spp.	6800	6	599
Scrupocellaria reptans	45	0	0
On west (wave-exposed) side of Carsaig Island:			
Patina pellucida	131	8	10
Spirorbis spp.	0	0	652
Scrupocellaria reptans	0	0	2

Between 6 and 12 meters below low water of spring tides the forest opens out, so that it was possible to walk between the *Laminaria* plants easily in spite of the rugged nature of the sea bottom; we therefore called this area the "park." In the park nearly every *L. Cloustoni* was entwined with a large unattached plant of the brown alga *Desmarestia aculeata.* Although 12 meters was the greatest depth to which we penetrated, it was possible through the misty water to see the park extending downwards much further on the steeply sloping bottom.

In extreme shelter from wave action *L. Cloustoni* is replaced by *L. saccharina,* or by a form, allied to this species, which is characteristic of the sea lochs of the west coast of Scotland (Kitching, 1935). The latter extends up to the lower margin of the zone of *L. digitata* in the extreme shelter of the small bay on the eastern side of the island, but southwards with increasing wave action it is progressively replaced from above by *L. Cloustoni.*

Associated Flora and Fauna

The canopy of *Laminaria* fronds is relatively clean of epiphytic algae in shallow water on the western side of Carsaig Island; instead it carries many colonies of the hydroid *Obelia geniculata* and the polyzoan *Membranipora membranacea,* and numerous gastropods, including *Patina pellucida.* On the more sheltered eastern side of the island *Obelia* and *M. membranacea* are much less abundant; they are practically confined to a narrow zone at the level of low water of spring tides, and they are absent from the very sheltered Bay (see map in Fig. 1); whereas in shelter filamentous epiphytes with their associated fauna, and the tube-worm, *Spirorbis* (spp.), and the polyzoan, *Scrupocellaria reptans,* may cover nearly all the space available on the *Laminaria* fronds. These same differences, though in lesser degree, may be observed at greater depths on the wave-exposed western side of the island. Here again it may reasonably be claimed that depth affords shelter from wave action.

The undergrowth of the sublittoral fringe consists mainly of the green alga *Cladophora rupestris* and the red algae *Chondrus crispus, Gigartina stellata,* and *Rhodymenia palmata.* Of these *Cladophora* and *Rhodymenia* also grow as epiphytes on the uppermost parts of the stipes of *Laminaria Cloustoni* in shallow water only, and small plants of *Rhodymenia* are sometimes found attached to the tips of *Laminaria* fronds in shallow water. The distribution of these algae is such as to suggest that they require a relatively high incidence of light.

The holdfasts and stipes of the *Laminaria Cloustoni* plants, except the upper parts of those nearest to the surface of the water, and the rock bottom between the holdfasts, are densely covered with an undergrowth of red algae, which includes *Membranoptera alata, Phycodrys rubens, Odonthalia dentata, Delesseria sanguinea, Ptilota plumosa,* and *Cryptopleura ramosum. Callophyllis laciniata* flourishes especially in the park, where its bright red fronds may be seen from afar. Apart from this, the composition of the undergrowth appears uniform from 2 to 12 meters. All these algae appear to favor a relatively weak illumination, a conclusion which is supported by photoelectric measurements reported later in this paper (Table II).

The tube-worms *Spirorbis* spp. and *Salmacina incrustans,* as well as various polyzoans and colonial tunicates, are abundant on the inner parts of the *Laminaria* holdfasts, even in places well exposed to wave action;

but the outer surfaces of these holdfasts are usually clean. The holdfasts shelter an extensive motile fauna, which will not be described in this paper. The composition of this fauna did not appear to be significantly influenced by depth within the limits of our exploration.

Recolonization of Denuded Areas of Laminaria *Forest*

In August, 1936 we were able to examine areas where the *Laminaria* forest had been cut down with shears 12 months previously. The old holdfasts had disappeared, and new *Laminaria Cloustoni* plants covered the area very densely, and had grown to a height of about 1 meter. The holdfasts were affixed very tightly to the rock. They were almost clean of epibiotic organisms, but a few specimens of *Spirorbis* were found on them. Some *Patina* were found on the stipes and fronds, but little else. The motile fauna associated with these young *Laminaria* plants was much poorer in numbers than that of the older forest, but was in general similar in constitution.

TABLE II

Position of sea photometer	Depth below water surface (meters)	Illumination cut off by seaweeds (per cent)
Under *Saccorhiza*, near *Callophyllis*	4.0	99.5
Among *Chondrus* and *Cladophora*, in zone of *Laminaria digitata*	1.5	48
At bottom, in old forest	3.9	98.7–99.4
Under dense new growth of *L. Cloustoni*, one year old	4.0	99.1–99.5
Under *Laminaria Cloustoni* in "park"	11	82–85
Under old *L. Cloustoni* forest	4.0	98.8–99.1

The first four observations listed above were made on August 13, 1936, between 12.25 and 1.00 P.M. G.M.T., under an overcast sky. The last two observations were made on August 22, 1936, between 2:08 and 3:15 P.M., under a sunny sky with light cirrhus cloud. On both days the wind was light, the water temperature about 12.5–12.6° C., and the air temperature 15–16° C. All these observations were made at station *A* (Fig. 1).

ILLUMINATION IN THE LAMINARIA FOREST

Apparatus and Methods

Measurements of the light intensity at a number of positions in the *Laminaria* forest were made photoelectrically, according to the general methods described by Atkins and Poole (1933). The light intensities in air and at a chosen under-water position were measured simultaneously and compared. Two "photronic" rectifier cells, made by the

Weston Electric Company, were employed. Separate galvanometers were used for the two cells, as this was found to be an advantage in changeable light, and also reduced the time during which the diver had to wait in the cold while readings were being taken. The cells were mounted in water-tight containers, and could be screened either with a flashed opal glass alone or with a selected color filter[1] underneath an opal glass.

The two cells were kindly calibrated for me by Dr. W. R. G. Atkins of the Marine Biological Laboratory, Plymouth. The calibrations were carried out by daylight under a variety of light intensities, with the cells mounted ready for use but dry, and under opal glass without color filters. Submersion under a thin film of water made scarcely any difference. For low light intensities, and with the low external resistance used, the relation between current and light intensity was approximately linear. For most purposes 10 ohm pattern L galvanometers made by the Cambridge Instrument Company were used, but for very low light intensities, as recorded in the depths of the *Laminaria* forest, it was necessary to use a 1000 ohm pattern LY galvanometer; however, at these low intensities of light the linear relation noted above was found still to hold in spite of the increased resistance of the external circuit.

It is recognized that the lux is not an ideal unit for present purposes; and that the measurement of illumination over a wide spectral range, with a photocell differentially sensitive with respect to wave-length, is not an entirely satisfactory procedure. However, it is believed that these objections do not invalidate the treatment of results which will be followed below.

Results

As a result of observations made with the opal screen alone, the light intensity in various situations in the *Laminaria* forest was found to be only a very small fraction of that in air. A comparison was then made of the light intensities within the forest and in open water at the same depth by using curves obtained in open water (Fig. 2). In this way the light intensity within the forest was expressed as a percentage of what it would have been had there been no forest, and from this was computed the percentage illumination cut off by the forest. A few typical results are given in Table II. In general the forest cut off about 99 per cent of the available light, and new growth one year old was about as effective. However, in the park, only 82–85 per cent was cut off by the seaweeds, and the illumination at the bottom was better than in the forest, even though the depth was greater. It is not possible to say to what extent this condition is general on the British coast.

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[1] Listed by the makers (Schott and Gen, of Jena) as BG 12 (blue, 2 mm. thick), VG 2 (green, 4 mm.), RG 1 (light red, 2 mm.), and RG 8 (dark red, 2 mm.).

The illumination at any one position in the forest fluctuates continually owing to the movement of the fronds overhead. Therefore it was

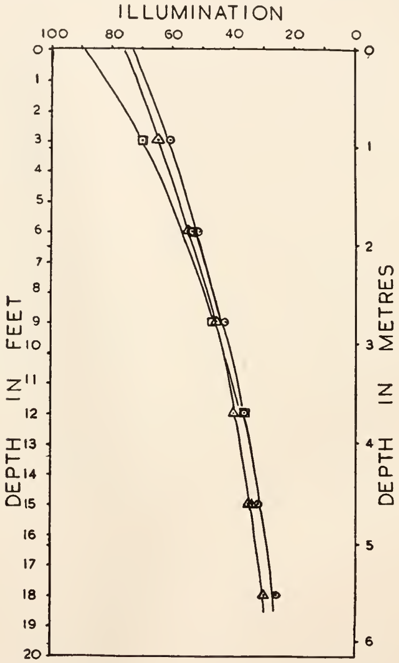

FIG. 2. Graph showing illumination at various depths in open water at position *A*, on the west side of Carsaig Island (see inset to Fig. 1). A plain opal glass, without color filters, was used. The illumination in air is rated as 100.

⊡ August 12, 1936 (transmission 79–81 per cent per meter).
△ August 13, 1936 (transmission 80–87 per cent per meter).
⊙ August 19, 1936 (transmission 81–85 per cent per meter).

found impossible with our equipment to investigate in detail the quality of the light in the forest, as compared with that in open water. However, since the *Laminaria* fronds are opaque and of a darkish brown color, it seems probable that not much light of any wave-length is either transmitted or reflected into the depths of the forest by the fronds; presumably most of that which reaches the depths of the forest passes between the fronds. Therefore, results obtained with the opal alone probably represent approximately the fraction of light of any wave-length which penetrates the forest, as compared with the illumination at that wave-length in the same depth of open water. It is therefore possible to estimate approximately the depths in open water at which the monochromatic illumination at various frequencies is equivalent to that in the *Laminaria* forest just below low water mark. For this somewhat rough and arbitrary procedure the illumination in the *Laminaria* forest has been taken as 1 per cent of that in open water at the same depth, and the transmission for light of various wave-lengths has been given the values obtained from Fig. 3, although indeed transmission may actually vary with depth. The results of this calculation are given in Table III, and are discussed further on (p. 334). The preferential transmission of green light is in accord with the results of many other workers in coastal waters (Knudsen, 1922; Atkins, 1926; Klugh, 1927; Clarke, 1936, 1939).

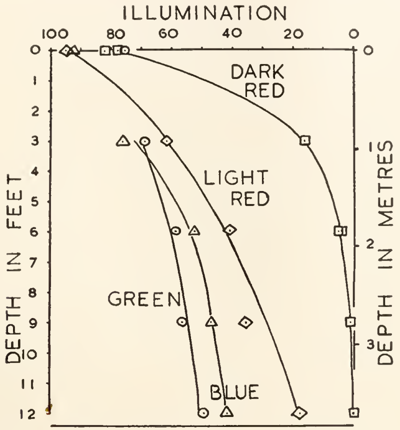

FIG. 3. Graph showing illumination at various depths in open water at position *A*, on the west side of Carsaig Island (see inset to Fig. 1). Color filters were used in addition to opal glass. The illumination in air is rated as 100. The observations were made on August 19, 1936.

Blue, 350–470 mμ, transmission 75–91 per cent per meter.
Green, 500–570 mμ, transmission 87–93 per cent per meter.
Light red, 620–700 mμ, transmission 53–65 per cent per meter.
Dark red, 700–750 mμ, transmission 27–28 per cent per meter.

DISCUSSION OF ZONATION

The zonation of plants and animals is very strongly marked in the littoral region of the British coast. The dominant algae are restricted very obviously to narrow belts along the shore; while the zonation of animals, although usually less precise, is fully shown by studies of population density and growth rate (Fischer-Piette, 1936; Moore, 1934). Littoral zonation may reasonably be attributed in the main to desiccation (Baker, 1910; Muenscher, 1915; Kanda, 1916; Johnson and Skutch, 1928; and Hatton, 1930, 1932), although in general decisive proof has not yet been given, and to a smaller degree to the effects of heat and light (Gail, 1919, 1922; Klugh and Martin, 1927). In response to the severe gradation of the controlling environmental factors, the zonation of organisms in the littoral region is sharply defined.

At the upper margin of the sublittoral region, along a strip of shore only uncovered at low water of spring tides, there is a peculiar and characteristic zone called appropriately by Stephenson *et al.* (1937) the "sublittoral fringe." The characteristic algae are probably restricted at their upper limits by desiccation, and at their lower limits by deficient illumination. Wave action is maximal here, and the larger brown seaweeds (*Himanthalia, Alaria, Laminaria digitata*), as is frequently pointed out, are well adapted by their pliable stipes to withstand sudden and violent stress. Perhaps of equal importance is the fact that at low tides they lie prostrate and so in the main escape desiccation. After low spring tides in very hot dry weather I have seen the fronds of *Laminaria digitata* around Carsaig Island scarred with dead patches where they had projected from the water. However, at greater depths these algae of the sublittoral fringe cannot compete with the erect and less pliable *Laminaria Cloustoni.* It is noteworthy that *L. digitata* fails to penetrate the true sublittoral region except as an epiphyte of *L. Cloustoni;* by this means it borrows the advantages of a tall erect stipe and achieves "a place in the sun." On the other hand, *L. Cloustoni*, though a true sublittoral form, may by its habit of growth expose its frond to the dangers of emersion at low water of spring tides, and is, in fact, quite probably limited by desiccation. The animals of the sublittoral fringe are restricted at their upper limits by exposure to air, but in general range downwards extensively into the shallow sublittoral, since they are less dependent on light than are the algae. However, in certain cases immersion may possibly be detrimental (Moore and Kitching, 1939).

In the sublittoral region proper the upper limits of distribution of organisms are determined by excessive wave action, and perhaps by excessive illumination in some cases; whereas the lower limits are set by deficient wave action and deficient illumination. The factors effective in controlling zonation below the sublittoral fringe are not steeply graded, and therefore zonation is not sharply marked.

The limitation of the vertical distribution of sublittoral organisms by wave action can be demonstrated readily, because in such cases these limits are raised where the coast-line provides greater shelter, depressed where it is more open to the waves. The upper limits of *Laminaria saccharina,* in its sea-loch form, and the lower limits of *Membranipora membranacea* and *Obelia geniculata* (see p. 327) are examples. The effect of wave action upon marine organisms is complex and obscure; and the amount of wave action necessary to support the existence of an organism may depend on other environmental conditions (Moore and Kitching, 1939). Apart from its destructive mechanical effect, wave action probably influences the settling of larval forms, promotes the circulation of planktonic food, disturbs sediment, and obliterates extreme fluctuations in the physical and chemical conditions of the water.

Limits of distribution determined by illumination, in contrast to those set by wave action, are likely to be relatively independent of the conformation of the coast-line over a small area, despite local variations in the loss of light at the surface of the water. The transition from the sublittoral fringe to the zone of *Laminaria Cloustoni* involves a decline in illumination at the sea botttom so great and so steep that none of the chief undergrowth-forming algae is common to both levels. However, within the broad *L. Cloustoni* zone, the illumination is by comparison almost uniformly dim, and over a range of 12 meters no case was found in which limitation could be attributed to illumination. Although the upper limits of the shade-loving undergrowth algae of the *L. Cloustoni* zone coincide with a sudden gradient of illumination, yet it is not clear whether these algae are restricted directly by excessive illumination, or by some other factor, such as a brief exposure during low equinoctial spring tides. It has been shown that in certain of these algae, *Delesseria sanguinea* and *Plocamium coccineum* (Moore, Whitley and Webster, 1923), and under certain conditions, the greatest photosynthetic activity takes place under moderate rather than very strong illumination; but for present purposes much more experimental evidence is required. It must also be remembered that this discussion is concerned throughout with organisms in competition, and that competition is likely to accentuate the sensitivity of these organisms to their environment (Beauchamp and Ullyott, 1932).

It is important to recognize that not only the quantity but also the quality of the light varies with depth. Green and blue light,—in coastal waters especially the former,—penetrate more readily than orange, red, and ultra-violet; and therefore with increasing depth they predominate to an ever-increasing extent (see the review by Clarke, 1939). Atkins (1926) has stressed the importance of this change in quality in relation to the vertical distribution of algae. In spite of certain obvious exceptions, it is in general true that green and brown algae are restricted to littoral and shallow sublittoral levels, whereas many red algae penetrate to greater depths. For instance, red algae have been dredged in the English Channel (Hamel, 1923) and off the Faeroes (Børgesen, 1908) from depths of 45 and 50 meters respectively, whereas the brown algae do not in general extend below 25 meters in Faeroese waters. At the depths to which red algae penetrate the light is mainly blue and green. It has been suggested (for references see Atkins, 1926) that by the nature of their photosynthetic pigments they derive the greater part of their energy from light of these colors; and this hypothesis may be applied with reasonable safety to algae growing at depths where green and blue light vastly predominate. However, various of these shade-loving red algae, *Phycodrys rubens, Delesseria sanguinea, Ptilota plumosa,* found at 40–50 meters off the Faeroes, are characteristic of the undergrowth of the *Laminaria* forest in the shallow sublittoral region, and are also found in sea caves (Børgesen, 1908; Rees, 1935). This implies a wide tolerance of variations in the spectral composition of the light. In spite of qualitative differences in illumination, there is clearly an ecological similarity between the shades of the *Laminaria* forest, the half darkness of caves, and the open sea bottom of the deeper sublittoral region. The incidence of green and blue light is probably of the same order in the deeper sublittoral and in the *Laminaria* forest (Table III).

TABLE III

Color of light	Blue	Green	Light red	Dark red
Range of filters (approximate)	350–470 mμ	500–570 mμ	620–700 mμ	700–750 mμ
Transmission per meter (see Fig. 4)	83%	90%	59%	27.5%
Depth at which monochromatic light intensity is equivalent to 1% of that in *Laminaria* forest near low water mark	25 meters	40 meters	10 meters	3 meters

Although this suggests that within the *Laminaria* forest the green and blue light alone are sufficient to support the shade-loving red algae, it still remains uncertain to what extent use can be made of light of other colors. It seems possible from the work of Klugh (1931) that some at least of the deeper water red algae, though able to make an exceptional use of green light, may nevertheless be able to utilize profitably a considerable range of the spectrum. Much more work is needed on this subject.

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SUMMARY

1. On the shores of Carsaig Island, Scotland, the sublittoral region is densely forested with laminarian seaweeds down to a depth of at least 15 meters, and probably down to a much greater depth.

2. At the upper margin of the sublittoral region, exposed to air at low water of spring tides, there is a characteristic " sublittoral fringe."

3. The dominant brown algae of the sublittoral fringe have pliable stipes, so that they lie flat at low water and escape desiccation. The dominant brown algae of the true sublittoral region have tall erect stipes, which hold up the fronds to the light.

4. The *Laminaria* canopy at depths of 1–6 meters cuts off about 99 per cent of the available light. At 6–12 meters the forest is less dense, and relatively more light penetrates. There is therefore a sharp decline in illumination between the sublittoral fringe and the *Laminaria* forest just below it; but within the forest the illumination changes much less over a considerable range of depth.

5. Wave action is considered to be maximal in the sublittoral fringe, and to decrease gradually with depth.

6. The undergrowth-forming algae fall into two clearly defined groups—one group confined to situations of high illumination, such as the sublittoral fringe and the upper parts of *Laminaria* stipes; and the other to dark places, such as the rock surface in the depths of the forest and the lower parts of the *Laminaria* stipes.

7. The undergrowth of the *Laminaria* forest is practically uniform in composition within a vertical range of about 12 meters, and probably more.

8. Vertical distribution is determined chiefly by wave action in the case of one laminarian alga, two polyzoa, one hydroid, and probably other organisms.

9. It was found that on artificially denuded areas new *Laminaria Cloustoni* plants grew to form a forest 1 meter high in 12 months.

10. It is concluded that whereas the steeply graded zonation of the littoral region is to be ascribed in the main to desiccation, the more gentle zonation of the sublittoral region depends on illumination and on wave action.

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FURTHER EXPERIMENTS IN CROSS- AND SELF-FERTILIZATION OF CIONA AT WOODS HOLE AND CORONA DEL MAR

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Of the many studies that have been carried out on the development of the eggs of marine animals, it is surprising how little attention has been paid to the development of 100 per cent normal embryos or larvae. Even in the case of the sea-urchin, where hundreds of experiments have been reported, the percentage of normal embryos is seldom, if ever, recorded. Many workers are contented with batches of these eggs in which the fertilization membrane is given off in practically all the eggs, but even then the number of such eggs that produce normal plutei is seldom stated. E. E. Just, almost alone, has emphasized the importance of using normal sea-urchin's eggs, and also the need of paying the strictest attention to the environment in which the eggs develop. Albert Tyler has also taken great care to use only normally developing eggs in his physiological experiments.

Sea-urchins brought back from the collecting grounds in crowded jars or in buckets to the laboratory or those kept in floating cars, or kept without food in aquaria are sometimes recognized as a source of abnormalities, but the condition of their eggs is, as a rule, ignored.

In the earlier work on artificial fertilization the occurrence of " swimmers " was often reported as indicating a successful result, but every embryologist knows that " swimmers " are abnormal embryos. Only as methods improved were normal plutei reported, but practically never was the percentage of normals given.

Most of those who have used sea-urchins are familiar with the fact that individuals are frequently met with whose eggs fail to give off a normal fertilization membrane, although the eggs appear to be normal. To what extent this is due to the eggs not being " ripe " or overripe is seldom known, even though in sea-urchins the polar bodies have been given off when the eggs leave the wall of the ovary and are presumably mature. In the starfish, on the contrary, the eggs free in the ovary still contain the large germinal vesicle which will disappear to form the polar spindle when the eggs are removed to sea water. It is well known, however, that such eggs often develop abnormally when fertilized. When a starfish spawns normally, the eggs, before extrusion, have matured, i.e., the germinal vesicle has disappeared, and it is recognized that such eggs give, as a rule, normal development. Of course, it is known that polyspermy in the sea-urchin is one of the factors of abnormal development, and this holds for other animals whose eggs are fertilized in the laboratory. Fortunately, in *Ciona* polyspermy is a relatively rare occurrence, and this source of abnormal development is practically eliminated. Nevertheless, sets of eggs that give 100 per cent two-cell stages sometimes give rise to some or to many abnormal embryos.

So far I have referred to what appear to be internal factors in the eggs themselves which cause abnormal development. What these factors are, aside from immaturity or over-ripeness, is generally unknown and has been little studied. On the other hand, it is well known that developing marine embryos are extremely sensitive to external factors such as temperature, salinity, impurities in the water, bacteria, etc. In forms that develop slowly (outside the parent), the chances of unfavorable conditions appearing are much increased, but even in rapidly developing forms, such as *Ciona,* external factors may also play a significant rôle. Fortunately, in this animal the completely formed tadpoles develop at 22° C. in fifteen hours or less, and it is not difficult to control the environmental factors during this time. But, as the following experiments clearly show, unless the eggs are thoroughly washed and the excess of sperm removed, abnormal development is apt to occur. Even then, however, different individuals may give quite different results when the environment is apparently the same for all. Perhaps this may be expressed by saying that the eggs of different individuals respond differently to the same environmental differences. If this is the correct interpretation, as the evidence at hand seems to indicate, it is evident that contradictions may appear when the eggs of different individuals are treated in the same way. This possibility makes the problem difficult, but repetitions of the same kinds of experiments have helped to clear away some of the apparent contradictions.

The most puzzling problem is the occurrence of both normal and abnormal larvae in the same culture of *Ciona.* The numbers may vary from one or two normals to 99.5 per cent normals. Sets of eggs of one individual fertilized by sperm of one other individual tend to give the same proportions, under external conditions that are the same, but even here, exceptionally, the ratios may vary. Different samples of eggs may account in part for these differences, even when an attempt is made to make the samples the same. Differences in the position of the eggs in the dishes may make for differences, but when few are present they space themselves equally on the bottom, and even when several thousand are present in a thin layer of water, practically 100 per cent may be normal. " Accidents " of development, such as the position of the cleavage planes, may be another factor, but it is impossible to say what causes these accidents,—whether they are internal or external.

The western *Ciona,* like the eastern one, sets free its eggs and sperm in the early morning in response to a change from darkness to light. In general, therefore, fewer eggs are found in the oviduct in the later morning than in the afternoon, and the former may be said to be younger than the latter, although all have formed the polar spindle before leaving the ovary. No difference has been observed between the younger and older eggs in respect to the development of normal embryos. When Cionas are brought into the laboratory and kept in running water, or in aerated water, the eggs may accumulate, in some individuals at least, until the oviduct is swollen with them. They may be two or three days old, yet produce as many normals as eggs from freshly-caught individuals. If the water is changed daily and kept clean, and only a few Cionas kept in the same jar, the eggs are good for at least three days.

My interest in the problem of abnormal development in *Ciona* is only secondarily concerned with the problems mentioned above. It became necessary to find out to what extent the abnormal development is due to internal factors on account of its possible relation to the genetic problem of self-sterility and cross-fertilization in *Ciona.* If abnormal development is due to inherited genetic factors, then its occurrence, if regular and internal, might be due to genetic lethals whose presence might bear on the main problem.

New Experiments with Ciona at Woods Hole, Mass.

The results of experiments with *Ciona* at Woods Hole which I carried out during the years 1904 and 1910, are in some instances more erratic or irregular than those obtained in recent years with what is said to be the same species on the California coast. This is true, moreover, of experiments that I carried out in 1904 at Coronado Beach, California, on *Ciona* which is undoubtedly the same type that is found at Corona del Mar. I was inclined to think that the methods of handling the eggs in the earlier experiments at Woods Hole might account for the differences, and therefore when I had a chance to test out the Woods Hole form during September, 1940 I carried out some experiments that I hoped would show whether the differences are due to the earlier technique employed, or to differences in the material itself. Two kinds of tests were carried out. There were five of the 5 × 5 cross- and self-experiments, and five experiments of a different kind.

In the 5 × 5 tests a larger amount of water was used than in the former experiments at Woods Hole; also the eggs were washed in one or two changes of water. The eggs were then concentrated in a small amount of the water, and 7 to 10 drops of the eggs were then transferred to Stender dishes containing 20 cc. sea water. The cleavages were noted, and after 20 to 24 hours the kind of development recorded. In all cases (100 in all) the cross-fertilized eggs gave 100 per cent cleavage. There were no failures to cross-fertilize. In the 25 selfed lots most gave no cleavage (except that in one case 95 per cent cleaved), but there were a few cleavages in some lots. It is to be noted that only 5 drops of sperm suspension were used, and the concentration of sperm was not large since the individuals were small.

The first lots came from a float in the seal-pool of the Fish Commission. There were at least a thousand Cionas on the float when removed; most of them were only half grown, but a few had eggs and sperm. Those reported in the first three sets came from this float; those in the two remaining sets came from the supply tank on the roof of the laboratory. These were also small individuals and only a few were mature or had enough eggs for the tests.

I. (Sept. 16, 1940.) All of the cross-fertilized eggs gave 99 or 100 per cent cleavage (except one that gave 50 per cent). The five selfed sets gave 0, 25, 25, 50, 40 per cent cleavage. The total number of eggs in the dishes (20 cc.) was between 50 and 100. After 24 hours it was found that three lots of eggs, viz., *c, d, e,* gave very abnormal embryos; one lot, *b,* gave all normal or late abnormal stages; and another lot, *a,* gave normal, late abnormal tadpoles, and early abnormal tadpoles. It was very noticeable that the eggs rather than the sperm determined the kind of devlopment that took place. Over and beyond this, however, there are differences in the different lots of eggs of the same set that seem to follow the sperm, or the combination of eggs and sperm.

II. (Sept. 16, 1940.) These Cionas came from the same float as the last. They had been kept in the laboratory in running water for one day. The 20 crossed lots gave 100 per cent normal 2-cell stages; rarely one or two of the selfed lots divided. Five drops of sperm suspension were used, and 15 cc. sea water. Taking the crossed eggs in the horizontal lines, *aB* and *aC* gave abnormal embryos, *aD* normal tadpoles and *aE* late embryos. The next lot, *b* eggs, gave all normal tadpoles as did the *c* eggs. The *e* and the *d* eggs gave nearly all abnormal embryos. The eggs appear responsible for the differences, and not the sperm, which was the same in each of the vertical lots.

III. (Sept. 18, 1940.) These came from the same source as I, but had been kept in running water in the laboratory for two days. Only a few of the Cionas had enough eggs for the experiment. The sperm duct was better. Five drops of sperm suspension were used, and the eggs were in 16 cc. sea water when fertilized. Most of the supernatant fluid was then drawn off the eggs, and 10 drops of eggs added to 20 cc. sea water in Stender dishes. There were 75 to 150 eggs in each dish (fewer in *d*). All 20 crosses gave 100 per cent regular cleavages, except *Ad* and *Bd*, that had a few irregular cleavages. None of the eggs selfed. The cross-fertilized eggs gave abnormal embryos that died young (stage *d* and *e*). (*See* Morgan, 1938*a*, p. 305.)

IV. (Sept. 19, 1940.) These Cionas came from the tank on the roof of the laboratory. The cleavage was not observed. The eggs were washed twice in sea water and 12 drops transferred to each dish (20 cc.). Five drops of sperm suspension were added to each. Four of the egg-lots gave normal and nearly normal tadpoles, one gave very abnormal embryos. Two dishes, *eA* and *eB*, contained only 10 cc. sea water and these gave very abnormal embryos, although the other two, *eC* and *eD*, gave normal and abnormal tadpoles.

V. (Sept. 21, 1940.) This lot also came from the tank and had been kept two days. The eggs were washed once; then eight drops were added to each Stender containing 20 cc. sea water, where they were fertilized (5 drops). Four of the lots had some normal and bent tadpoles, but even these dishes had, for the most part, abnormal embryos; one lot had only very abnormal tadpoles. It is not evident in this set whether the great variability in each dish and between different dishes is due to the condition of the eggs or to the presence of the sperm in the dishes. One dish of selfed, *eE*, gave 113 short abnormal tadpoles, 12 late abnormal and 4 abnormal embryos; the other selfed lots had nearly all unfertilized eggs.

It is quite evident even from these few experiments that practically 100 per cent cleavage occurs if the eggs are washed and not too much sperm added. Evidently these Cionas at Woods Hole behave in the same way as those at Corona del Mar. The cleavage irregularities in the 1904 and 1906 experiments must have been due to handling. It is also clear here that despite the occurrence of the normal 2-cell stage, the eggs of several of the individuals gave abnormal embryos and abnormal tadpoles.

Some further tests were made on some of these Woods Hole Cionas in order to study the effect of external conditions on development.

(Sept. 19, 1940.) The eggs were washed, then cross-fertilized (*A* by *b* and *B* by *a*) by 5 drops of sperm suspension. All eggs in both sets cleaved. In the two-cell stage the eggs (5 drops) were transferred to 10, 20, 40 cc. of sea water. All sets gave abnormal embryos. These results are clearly due to the condition of the eggs and are nearly the same as two of the lots in the 5 by 5 tests of the same date. The other three lots of the same 5 by 5 test gave mostly normal tadpoles.

(Sept. 21, 1940.) Two small Cionas from the tank were used. The eggs were washed once, and 10 drops were transferred to 10 and 20 cc. sea water, where they were fertilized (5 drops). *A* by *b,* in 10 cc., gave late abnormal embryos, and in 20 cc. 49 nearly normal tadpoles and 19 normals. *B* by *a,* in 10 cc., gave the same result as above; and in 20 cc. there were 85 normals and 3 abnormal tadpoles. Here 20 cc. gave distinctly better results than 10 cc. It will be noted that 5 drops of the sperm suspension were left in each dish.

(Sept. 21, 1940.) The water was changed once on the eggs, then they were cross-fertilized (5 drops). The supernatant water was then largely removed and 8 drops of eggs added to each dish of 10 and 20 cc. sea water. The *A* by *b* in 100 cc. sea water gave 95 per cent normals; and in 20 cc. gave 99.5. But *B* by *a* in 10 cc. gave 145 abnormal embryos and in 20 cc. gave only 9 normal tadpoles, 6 bent and 109 abnormal tadpoles. Here also the results were a little better when more water was present, but there was a striking contrast between the reciprocal crosses.

In a repetition of the same experiment on the same date, *A* by *b* eggs in 10 and in 20 cc. sea water gave abnormal tadpoles; *B* by *a* eggs in 10 cc. gave abnormals as before, but in 20 cc. gave 393 normals, 109 twisted tadpoles and 62 eggs or young embryos.

(Sept. 24, 1940.) The eggs were washed once. Ten drops of eggs were then transferred to 20 cc. sea water in two dishes (*a* and *a′*). Here they were fertilized with 5 drops of sperm suspension. After 15 minutes the water was removed from one dish (*a′*) and replaced by new sea water. All sets gave 100 per cent cleavage. The embryos in *a* died young, but *a′* gave 6 nearly normal, 40 crooked tadpoles, and 76 very abnormal tadpoles. Evidently the latter set went further than the former (in which the sperm was left). The reciprocal cross, *B* by *a,* gave somewhat better results. In *b* there were 11 normal, 12 crooked, and 59 very abnormal tadpoles. In *b′* there were 14 normal, 14 crooked and 49 very abnormal tadpoles. The results were the same in *b* and *b′*, and somewhat better than in *a* and *a′*.

It is very noticeable that with the better technique practically all the cross-fertilized eggs in the set of experiments gave 100 per cent cleavage, but nevertheless there was a high percentage of abnormal development. The latter can only be ascribed to internal conditions in the eggs or sperm, probably in the former. The Cionas were just reaching maturity, i.e., about half normal size. The test was transparent and thin. It seems safe then to ascribe the abnormal development to immaturity of the animals although the eggs had every appearance of being normal. There is here a rather sharp contrast between the normal behavior of the eggs and sperm with respect to cross- and self-fertilization, and the failure of many of the fertilized eggs to produce normal tadpoles.

Review of Earlier Work

In the summer of 1903 (see *Jour. Exper. Zool.,* 1904) I made four 5 by 5 experiments on Cionas at Woods Hole, Mass. In one of them practically all the cross-fertilized eggs segmented. In another the sperm completely failed in two cases and practically in a third (giving 30 per cent in one case), although the eggs were good as shown in the other two cases. In a third test the sperm was not very good (except in one case). In a fourth test the sperm was not good in one case (except with one set of eggs where it gave 75 per cent cleavage). It seems now evident that the failure to cross-fertilize was due to the sperm suspension, and not to the conditions under which the tests were made.

Six years later (1909. See Roux's Archiv., 1910) abundant material was available at Woods Hole and fifteen 4 by 4 tests were carried out (180 crosses in all). Of these only two gave uniformly good results. In some, the four lots of eggs were obviously poor, in others the sperm, but in all of them (except two) there were inconsistent results as though certain combinations were incompatible, but in the light of the results with the California type of *Ciona* it seems highly improbable that these failures to cross-fertilize (i.e., to cleave) were due to the presence of individuals with identical genetic composition, and there is no evidence that the results were due to differences in the environment.

In 1932 (Sept. 19) a few large Cionas were brought to me at Woods Hole. I tried out one 5 by 5 experiment for the cleavage. All cross-fertilized eggs gave nearly 100 per cent (one 95 per cent). The selfed lots gave 0, 0, 8, 0, 4 per cent. This is the same kind of result as with the California type. Some other eggs were treated with crab juice (*Callinectes*) for four hours, and then selfed. They gave practically 100 per cent cleavage. When treated with acid sea water and selfed they gave 100 per cent cleavage. In both respects these eggs agree with the California Cionas. It is noticeable, in comparison with the later (1940) experiments at Woods Hole on small immature Cionas, that these Cionas were large, and the results were uniform and good.

In the summer of 1904 I spent a couple of weeks at Coronado Beach, California, at the abandoned yacht club that had been used earlier as a marine station of the University of California. There were quantities of large Cionas on the float at the station. The laboratory room was very warm during the daytime and the glassware insufficient. Only small saltcellars were available for the eggs, etc. The results of eight 5 by 5 tests were published in 1905. On the whole, the results for cleavage were poor and irregular. There were at least 17 cases where the sperm gave no cross-fertilization or very little, and almost as many cases where the eggs were poor, in the sense that they did not cleave although they had every appearance of normal eggs. That the heat in the laboratory was not the cause of the failure to cleave was evident since in every set there were cases of normal (100 per cent) cleavage, but the heat may account for the almost entire failure to give normal embryos. The dishes were treated uniformly, although owing to the abundance of sperm, too much may have been used, which, while not affecting the cleavage, would affect the development. The only explanation I can offer is that the temperature of the water in the basin around the float had injured the reproductive cells before the Cionas were removed.

The Causes of Variability of Normal and Abnormal Development

From March to the end of June and again during October and January, 1940–41, I repeated, with improvements in the technique, some of the earlier experiments that had been made to find what conditions determine whether normal or abnormal development takes place, whether internal or external.

It had become evident from previous experiments that better, i.e., more uniform results, take place if the eggs are thoroughly washed, then fertilized with a few drops of sperm suspension, most of the supernatant fluid removed, fresh water added, and, after the eggs have settled, most of this water also removed leaving only enough to supply a drop of eggs to each of the dishes in which the eggs are to develop. An experiment of this kind was made (Oct. 19) at Corona del Mar with Cionas freshly brought in. The eggs were washed, and 10 cc. of fresh sea water added. They were cross-fertilized by 5 drops of sperm suspension, and after 10 minutes the supernatant fluid was drawn off (except 20 drops). One drop of these fertilized eggs was added to 10 cc. sea water (in each of 10 dishes). The eggs were brought back to Pasadena in closed Stender dishes, except the last four that were brought back in closed vials. *A* by *b* gave the following figures:

94, 97, 99, 93, 99, 99, 99, 99.5, 99.5, 99.5 per cent tadpoles

The reciprocals, *B* by *a,* gave a much smaller percentage of normals:

49, 46, 28, 24, 3, 59, 38, 31, 35 per cent tadpoles

The latter figures are less than half the former and there is more variability, although the external conditions were made as like as possible. The differences in the two cases seem to depend on the eggs or on the sperm (or both). The amount of sperm carried over with one drop of fertilized eggs must have been too small to affect the results, even if there was some initial difference in the sperm suspensions.

The next day (Oct. 20) the same experiment was carried out with fresh eggs from the adult Cionas that had been brought to Pasadena. The results were very uniform, giving 99 per cent throughout (except one, 95 per cent). Repeated four times (Oct. 20–21) all gave about 100 per cent normals.

In order to test further (Nov. 3) whether the transportation of segmented eggs in closed Stender dishes gives the same results as do those left at Corona del Mar, since in one case not reported here some of the former were abnormal, the eggs of fresh Cionas were washed twice, then fertilized, and after 15 minutes the supernatant fluid was taken off (except about 20 drops). Then one drop of these fertilized eggs was added to each of 20 Stender dishes (20 cc.). Ten of these dishes, *A* by *b*, were taken (after 4 hours) to Pasadena. The percentages of normal embryos were: 52, 74, 80, 95, 91, 52, 96, 0,[1] 55, 80 per cent; average 75.0 per cent. The other ten dishes left at the shore gave: 100, 99.5, 100, 99.5, 99.5, 0,[1] 60, 100, 70, 100 per cent; average 92.1 per cent. In each set there was one dish that gave no normals; both had dirt in them and were disregarded. Those left at the shore had a higher level of normals, which is probably not significant. Difference in temperature was not involved here, since the car was cool. Six dishes of the reciprocals, $B \times a$, were also tested in the same way. Those taken to Pasadena gave: 93, 87, 87, 93, 92 per cent. These average a little better than the corresponding *A* by *b*. Those left at Corona del Mar gave: 100, 99, 99, 99.5, 93 per cent. The differences are probably not significant since the percentages are estimates only.

The following experiment was made with Cionas (Nov. 11) that had been kept for two days (with change of water). The eggs were washed twice and all fertilized by five drops of sperm suspension. After 15 minutes the supernatant water was changed, and one drop of eggs put into each of ten Stender dishes containing 15 cc. sea water. The reciprocal test was treated in the same way. The percentages in the first dishes were: 68, 69, 69, 73, 79, 80, 70, 78, 76, 80 per cent. The abnormals were of two kinds, viz., late abnormal tadpoles and early abnormal embryos in about equal numbers. The reciprocal ten dishes gave: 96, 97, 97, 96, 96, 98, 97, 99, 97, 99 per cent. The percentages are higher here than in the other ten dishes, and there is less variation. Many of the eggs (4224, and 4624) were left over in the original Syracuse dishes containing little water. Despite the small amount of water and the large number of eggs, the percentages (A by $b = 80$ per cent, B by $a = 98$ per cent) were not very different from those above.

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[1] All were late abnormal tadpoles. The contrast with the others is not so great as it appears to be.

The Cionas used in the next experiment had been brought to Pasadena (Oct. 12). Two days later (Oct. 14) the eggs of one of them were distributed in 10 dishes (10 cc. sea water), fertilized by sperm of another individual (one drop). The sperm was left with the eggs in this case, but only one drop to each dish. The actual counts were:

Normal Tadpoles	163	138	196	188	65	43	89	104	108	80
Abnormal tadpoles:	10	2	2	2	4	1	1	—	1	—
Abnormal embryos:	99	112	137	157	55	86	87	102	71	75

Counting both kinds of abnormals together, the percentages of normals are 60, 55, 58, 54, 52, 33, 50, 50, 60, 52. The variability is not much, but the percentages of normals are low.

The reverse (not reciprocal) test, with eggs that came from the same individual that supplied the sperm above, fertilized by sperm from one other *Ciona,* was much the same, but somewhat better:

Normal Tadpoles	45	78	50	65	102	70	100	124	59	165
Abnormal tadpoles:	29	12	12	2	8	29	11	13	30	16
Abnormal embryos:	15	13	8	8	21	22	18	22	16	30

The percentages are 50, 75, 71, 87, 78, 58, 78, 78, 56, 78. The percentages of normals are low in both these tests, and not obviously environmental.

Since the experiments in which reciprocals are tested often show differences in the proportions of normal embryos, some further tests (Jan. 10 and 14) were made in which the eggs were first washed in two changes of water, and, after fertilization, the supernatant fluid was changed twice. At the two-cell stage one drop of eggs was added either to 10 cc. in a Syracuse dish or to 20 cc. in a Stender. The dishes had been washed with a weak hydrochloric acid solution, put into running tap water for two hours and then rinsed in distilled water. In one case (10 cc.) A by b gave 100 per cent; B by a 80 per cent. In another case (10 cc.) A by b gave 90 per cent and B by a 80 per cent. There were two dishes of each which gave the same results. Also the many left-over eggs in each case gave approximately the same ratios. Furthermore, some of the unfertilized eggs kept in another dish and fertilized by sperm of a third individual gave about the same kind of results. The same statement may be made for two other reciprocal pairs in 20 cc. sea water. These results are in line with previously recorded cases showing often different proportions of normal tadpoles in reciprocals.

Finally another test (Jan. 13) of the same kind with the same preliminary precautions was made in which ten similar dishes (20 cc.) were made up of the cross and ten of its reciprocal. This experiment was made as a further check on different dishes of the same sort which should give more precise confirmation as to the reliability of the experiments. The actual counts are given in Table I. In the first column the number of normal tadpoles is given; in the second the number of abnormal tadpoles which were crooked or twisted; and in the third the abnormal embryos that had died at an early stage without evident differentiation.[2]

TABLE I

		A by *b*					*B* by *a*		
	Normal	Abnormal Tadpoles	Abnormal Embryos	Percentage		Normal	Abnormal Tadpoles	Abnormal Embryos	Percentage
1	247	74	12	74	1	54	23	7	64
2	138	2	11	91	2	22	0	4	85
3	275	17	11	90	3	17	8	2	63
4	137	11	11	86	4	40	7	1	83
5	167	0	9	95	5	88	0	9	91
6	184	5	7	94	6	28	0	1	96
7	332	8	17	93	7	32	0	1	97
8	63	102	4	37	8	1	205	9	0.5
9	111	4	8	90	9	96	0	3	97
10	53	111	11	30	10	87	0	9	90

Several of the ratios in Table I call for comment. The ratios are made up of the normals against the two kinds of abnormals taken together. Except for 8 and 10 in *A* by *b*, where the ratios are 37 and 30 per cent, the other ratios are nearly the same. In these two the low ratio is due to an excess of "abnormal tadpoles." Again, in *B* by *a* the ratios are nearly the same, except for 1, 3 and 8. Here also the low ratios are due to excess of abnormal tadpoles and not to younger stages, i.e. "abnormal embryos." Number 8 had only one normal tadpole. The total average for *A* by *b* is 77.2 and for *B* by *a* 76.9. If number 8 is eliminated, the difference between the two crosses is small. I am inclined to think that the "abnormal embryos" are due to internal factors. Polyspermy is rare and only a part of this kind of embryo can be assigned to it; the others are probably due to failure to cleave normally in an early stage. On the other hand, most of the "abnormal tadpoles" seem to be due to environmental factors, especially the extreme cases. The individuals of this group have for the most part developed nearly normally to a late stage and their defects are due to failure to straighten out at the time of hatching. Nevertheless, since in the less extreme cases of low ratios the large majority of the eggs have developed normally, the environmental factors must have been nearly normal, which would mean that some of the eggs were more sensitive to outside agents than are others. This conclusion is borne out in some of the earlier experiments where more normals were present when the eggs developed in a larger volume of sea water, which would tend to dilute those external agents that act injuriously. In the earlier experiments, where reciprocal crosses were made, differences in the sperm suspension was probably one of the factors that would account for the difference between the cross and its reciprocal, but in these later experiments that factor is practically eliminated by washing the eggs and removing the supernatant sperm suspension. The eggs were thoroughly mixed and one drop only of the eggs was added to each 10 or 20 cc. of sea water. It is unlikely then that the differences in the dishes of the same composition are due to factors of this kind, and can only be ascribed to differences in the dishes themselves. A number of further tests of reciprocals were made which need not be recorded here, but which confirm the conclusion that reciprocals may consistently give different ratios which can only be due to internal factors in the eggs. Since no definite ratios appear in the data, it is highly probable that there is no specific lethal genetic factor involved.

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[2] The left-over eggs (about 2004) of *A* by *b* gave about 99.5 per cent normal and 5 per cent early embryos, and *B* by *a* (about 700 eggs) gave about 80 per cent normals, 15 per cent abnormal tadpoles and 5 per cent abnormal embryos. These results are about the same as those in Table I, where fewer eggs and much more water were present. In addition a few (150 and 100) eggs of *a* were fertilized each by sperm from a third individual, and gave, respectively, *C* by *a* about 95 per cent normal and *C* by *b* 95 per cent normals, which are as good percentages as the best in the table.

The Development of Eggs from Different Levels of the Oviduct

In order to find out whether eggs from different regions of the oviduct give different percentages of normals, the following experiments were made. A very large *Ciona,* freshly collected (Nov. 23, 1940), was opened and the long oviduct was tied off by two ligatures into three sections, one near the outlet, one in the middle, and one next to the ovary. The first section would be expected to contain the oldest eggs, and the section next the ovary the eggs most recently set free from the ovary. Each section was opened separately. The eggs were washed twice, cross-fertilized with five drops of sperm suspension, which was largely removed when the eggs had reached the two-cell stage. Fresh water was added and then drawn off and replaced by fresh water (10 cc.). The first and second sections gave 100 per cent normal tadpoles. There were about 2,000 eggs in each dish. The third section (nearest the ovary) gave 85 per cent normals and 15 per cent bent and coiled tadpoles; 800 eggs in all. These differences are not significant as later results showed.

The experiment was repeated the next day (Nov. 24, 1940) on Cionas brought to Pasadena. The first and second (bracketed) are reciprocals as are also the third and fourth.

Section 1	Section 2	Section 3
80	90	99
99	70	70
99.5	100	100
99.9	100	100

There were from 800 to 1200 eggs in a dish (Syracuse) in only 10 cc. water, yet the percentage of normals was very high. There is no apparent difference in the percentages from the three different regions. It follows that different percentages of normals and abnormals in other experiments, when the eggs from the oviduct were used, are not due to age differences in the oviducal eggs.

The Cleanliness of the Glassware

The failure to get uniform results relating to normal development when the conditions of the experiments have been made as uniform as possible drew attention finally to the cleanliness of the glassware. As a rule the dishes were used for no other purpose, and were washed in tap water after each experiment, and drained, partly inverted, and not used again until dry. It was noticed that whenever drops of water had remained sticking to the glass and evaporated there a slight residue or stain was left. There was not sufficient reason to suppose such a minute amount of salts could affect the results, but, as a check, the dishes in one test were put into cleaning fluid, washed in running tap water and rinsed in distilled water, and used at once (Dec. 1). In four sets over 95 per cent normal tadpoles developed, but in one set the tadpoles failed to come out of the membrane. Clearly all the cleaning fluid had not been removed in this set. As a check the experiment was repeated (Dec. 2), omitting the cleaning fluid, and washing with distilled water and the double distilled water used in the last experiment. Normal tadpoles developed, 95 to 100 per cent. Finally, 10 dishes were scrubbed with trisodium phosphate, washed thoroughly in running water and drained. Washed eggs in the two-cell stage were added to 10 cc. sea water in Stender dishes. All gave late abnormal embryos. There were eight control dishes washed only in tap water which gave normals (most of them 100 per cent). When the same kind of experiment was repeated, but, after scrubbing the dishes in trisodium phosphate, they were washed, rinsed in 10 per cent HCl, and washed again, they gave 90 to 100 per cent normals, as did the controls washed in tap water, as also did some other dishes rinsed in HCl, and washed in tap water. As a further check other dishes were scrubbed in the trisodium phosphate and very thoroughly washed in running tap water. These also gave 99 to 100 per cent normals. It is evident from these experiments that the developing embryos are very sensitive to even traces of the chemicals used here in cleaning the dishes. The ordinary procedure of washing in tap water gives the best results, as a rule. Any salts that happen to remain as stains do not interfere with normal development. In fact, unless the utmost care is taken in removing the chemical material used to clean the dishes, there is more risk of causing abnormal development than from ordinary tap water.

Summary

A repetition of some of the earlier work on Cionas at Woods Hole (in 1904, 1905, 1910), with improvements in the technique that the later work at Corona del Mar had shown to be important, has made it clear, so far as cross-fertility and self-sterility are involved, that the Woods Hole type gives the same results as the California type. The very eccentric results concerning normal and abnormal development shown in the earlier experiments appeared again, and were found not to be due entirely to differences in technique, but to differences in the eggs of the Cionas themselves, connected, in part at least, with immaturity of the animals, even when their eggs and sperm appeared to be normal, and when the eggs cleaved normally, at least into two cells.

Some of the later work, carried out at Corona del Mar in 1940 when greater care was taken in removing the egg-water (by washing the eggs) and also in removing the excess of the sperm suspension, is reported. In most cases the eggs were fertilized en masse, and, after washing again and removing the excess of water, a drop or two only of the eggs was added to the sea water (10 to 20 cc.) in Stender dishes. The reciprocal cross was made in the same way. It was found that there is a marked tendency for all the dishes of the same cross to give closely the same percentages of normal tadpoles, but there were occasional dishes that gave more extreme variations (usually more abnormals). These are due to environmental factors. The cross, when compared with its reciprocal, frequently gives different percentages of normal development when the external conditions (water and dishes) are as nearly the same as possible.

Evidently, then, the eggs of different individuals in reciprocal crosses may give different percentages, although from a genetic (chromosomal) point of view the two are, on the average, identical *after fertilization.* It seems to follow, then, that this difference must lie in the cytoplasm of the eggs which in each case has been formed under the diploid condition of the eggs. There is also other evidence supporting such an interpretation. It should be pointed out, however, that, as a rule, the percentages of the two reciprocals are the same or nearly so. When they differ more markedly there are no definite ratios between them, so far as the observations go.

A number of experiments were also made to test whether differences in the dishes, used in the experiments after washing in tap water and drying, are responsible for the variability sometimes found in the same series. There is no evidence that this is the case if the dishes have been carefully washed and drained. On the other hand, if they have been cleaned by the ordinary chemical treatments there is evidence of effects on the development unless great care is taken to remove every trace of the cleaning fluid.

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RELATION OF THE SIZE OF " HALVES " OF THE ARBACIA PUNCTULATA EGG TO CENTRIFUGAL FORCE

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The centrifugal force used in my previous work (1932–1940) to obtain " halves "[1] of the egg of *Arbacia punctulata* has been about 10,000 × g. With this force, the egg breaks into two halves:—a (light) white half containing oil, nucleus, clear layer, mitochondrial layer and some yolk; and a (heavy) red half containing most of the yolk and all the red pigment granules. A small electric centrifuge was used, whose highest speed is approximately 10,000 R.P.M., or 160 R.P.S. The hematocrit tubes used to hold the material were 6.5 cm. long with an inside diameter of 0.35 cm. When placed in the arms of the centrifuge, the radial distance from the axis of the centrifuge to the position of the eggs while rotating was 10 cm. The centrifugal force is computed from the equation

$$F = .04 \times R \times (R.P.S.)^2$$

where R = radius in cm. and the force is expressed in times gravity.

Approximately this same force (10,000 × g) has been used by others to obtain the red and white halves for studies on permeability to water (Lucké, 1932), respiration (Shapiro, 1935), indophenol oxidase (Navez and Harvey, 1935), peptidase (Holter, 1936), and dehydrogenase (Ballentine, 1940). The segregation of different materials into the two halves has furnished a nice means of determining the location of various cell activities.

In any one batch of eggs, the white halves (and the red halves) are quite uniform in size. The *relative* size of the two halves throughout many experiments has been remarkably uniform; the white half is slightly greater in diameter than the red, and about one and one-third times greater in volume, with the force of 10,000 × g at 23° C. This I will call the standard force, since it has been so widely used.

Much greater centrifugal forces can be obtained with the air turbine.

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[1] The term " halves " is used incorrectly but purposely because there is no word to express two, and only two, unequal fractions of a whole, constant in size. The term " fragments " used by some writers implies variability in size, as well as in number.

The small lucite tubes used measure 1.4 cm. in length and have an inside diameter of 0.3 cm. The radius from the center of the rotor to the position of the eggs while rotating is 1.2 cm. The highest speed obtainable with the rotor used was 1,500 R.P.S., which gives a centrifugal force of about 100,000 × g. Somewhat lower forces are obtained by using lower speeds of the air turbine; the lowest speed of the air turbine gives a slightly greater force than the highest speed of the electric centrifuge. A very low force can be obtained with a low speed of the electric centrifuge. There is then, a range in the centrifugal force available of from 4,000 × g, just sufficient to break the eggs, to 100,000 × g.

The relative size of the two half-eggs varies with the centrifugal force used to break the egg in two. As mentioned above, with the standard force of 10,000 × g, the white half is one and one-third times the volume of the red half. With a force of 60,000 × g, obtained by a medium speed of the air turbine, the two halves are equal in size. With a force of 80,000 × g, the size of the two halves is the reverse of that obtained with the standard force, the red half being now one and one-third times the volume of the white. With the greatest force obtainable, 100,000 × g, the red half is five times the volume of the white. The white half is therefore very small and contains only the nucleus, some oil and a little of the clear matrix. Correspondingly, with the lowest force that will break the egg in two, 4,000 × g, obtained with a low speed of the electric centrifuge, the white half is five times the volume of the red. The greater the force, the larger the (heavy) red half and the smaller the (light) white half.

TABLE I

Arbacia punctulata. Size and force

		Diameter (μ)		Volume (μ^3)		Ratio (approx.)
Whole egg		74		212,000		
Minutes to break	Force (× g)	W	R	W	R	W : R
20	4,000	70	41	180,000	36,000	5 : 1
4	10,000	62	56	125,000	92,000	4 : 3
1	60,000	59	59	107,000	107,000	1 : 1
3/4	80,000	56	62	92,000	125,000	3 : 4
1/2	100,000	41	70	36,000	180,000	1 : 5

The time required to break the eggs apart at the different forces is, of course, different; it requires 20 minutes with the lowest force, and only one-half a minute with the highest force. In Table I will be found the forces used, the sizes of the two halves, their approximate ratio, and the time necessary to break approximately one-half the eggs into the two parts at 23° C. With the very high speeds of the air turbine, some inaccuracy and variability obtain, owing to the fact that the turbine must be speeded up and slowed down somewhat gradually, and the interval of full speed is so short (½ min.).

Together with the difference in size of the two halves with different forces, there is also a difference in the degree of stratification of the egg just before breaking. With a low force applied for a long period—20 minutes—the granules are entirely segregated into their respective layers according to their density, and well packed. With a somewhat higher force, the standard force, where 4–5 minutes are necessary to break the eggs apart, the granules are segregated into well-defined layers, but they are not so well packed, especially the pigment granules. With the higher forces of the air turbine, requiring only ½ to one minute, the egg breaks apart before the granules are entirely segregated so that there is no very definite stratification. Photographs 1–10 show the whole eggs centrifuged with different forces, just before breaking in two, and the two halves into which they break. With the greatest force, the materials are so poorly segregated (Photograph 5) that the red half contains some of all the materials present in the whole egg except the nucleus. By re-centrifuging the red half at the standard force, a new stratification is obtained just like that of the whole egg at this force (Photograph 12; cf. Photograph 2). A similar red half obtained with the standard force, has, when re-centrifuged with this same force, no oil or mitochondrial layer, these materials having been completely segregated out into the other half in the first centrifuging (Photograph 11).

It will also be noted that there is less elongation of the egg prior to breaking, with the higher forces.

If centrifuged slowly at first with a low speed of the electric centrifuge until well stratified, and then transferred to the air turbine and

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PLATE I

Explanation of Figures

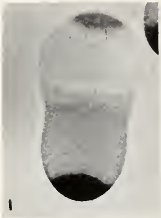
(Magnification, 275 ×)

Photographs 1–5. Stratification of *Arbacia* eggs at different centrifugal forces, just prior to breaking in two.

Photographs 6–10. The two halves into which the eggs break at different forces.

Photograph 11. A red half obtained with 10,000 × g (Photograph 7) re-centrifuged at 10,000 × g. Control to Photograph 12.

Photograph 12. A red half obtained with 100,000 × g (Photograph 10) re-centrifuged at 10,000 × g. Note re-stratification similar to original egg at this force (Photograph 2); nucleus, of course, absent. Mitochondrial layer stained with methyl green.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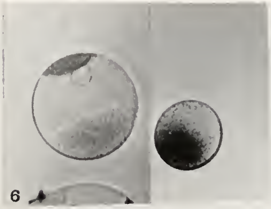
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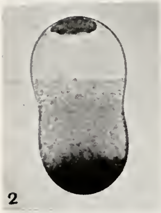
4,000 x g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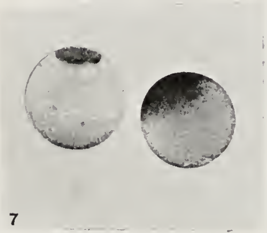
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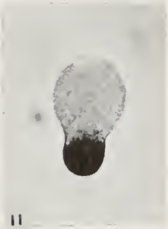
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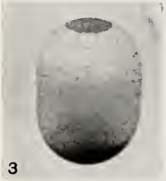
10,000 x g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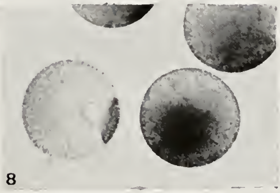
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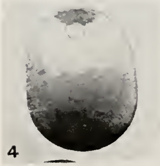
60,000 x g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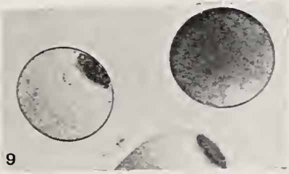
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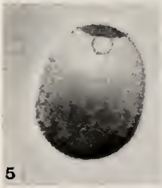
80,000 x g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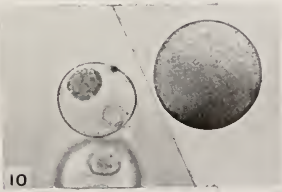
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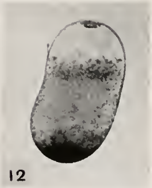
12

100,000 x g

PLATE I

centrifuged rapidly, the eggs break in just the same way as though they were kept at the low speed; that is, the white half is much larger than the red.

As has been noted previously (1936), several irregular batches of eggs occur each summer in which the break is quite different from the ordinary though the eggs themselves appear no different. In these eggs, at the standard force, the white halves are very large, and the red halves are very small (Photograph 16). The red halves ($D = 28.5\,\mu$; Vol. $= 12{,}000\,\mu^3$) are only about one-seventeenth the volume of the white, considerably smaller than those obtained from normal lots with the lowest force capable of breaking the egg, and even smaller than the pigment quarters obtained by breaking apart the usual red half with the standard force (Harvey, 1936, p. 103). These small red halves contain little besides the pigment granules which have been determined by E. N. Harvey (1932) to form 5.5 per cent of the materials in the egg. With a medium speed of the air turbine (60,000 × g) the red halves of the irregular batches are much larger, but still smaller than the whites; the two halves are of about the same size as those from ordinary batches at the standard force (Photograph 17, cf. 13). With the highest speed of the air turbine (100,000 × g), the two halves are about equal (Photograph 18, cf. 14). These irregular batches, then, show the same increase in size of the red halves with increase in centrifugal force as the normal batches, though starting from a different point.

It might be of interest to mention that these irregular batches can be made to break into the normal sized halves with the standard force, by keeping them in hypotonic sea water (80 per cent sea water, 20 per cent distilled water) for an hour and then centrifuging them in a sugar-sea water solution of the same tonicity.

The same general relationship between centrifugal force and the size of the half-eggs was found previously (1933) for the Naples sea urchins, *Arbacia pustulosa, Sphaerechinus granularis* and *Paracentrotus lividus,* within a much more limited range of forces then available. For the egg of *Parechinus microtuberculatus* (from Naples) and *Tripneustes*

PLATE II

Explanation of Figures

(Magnification, 125 ×)

Photographs 13–15. Normal eggs broken at different forces. Control to Photographs 16–18. The slightly larger sphere at the middle right in Photograph 15 is a whole egg, the others are halves.

Photographs 16–18. Irregular batch broken at same forces as 13–15.

Note uniformity in size of halves at any one force. There is usually a slight irregularity in normal batches at the highest force (Photograph 15).

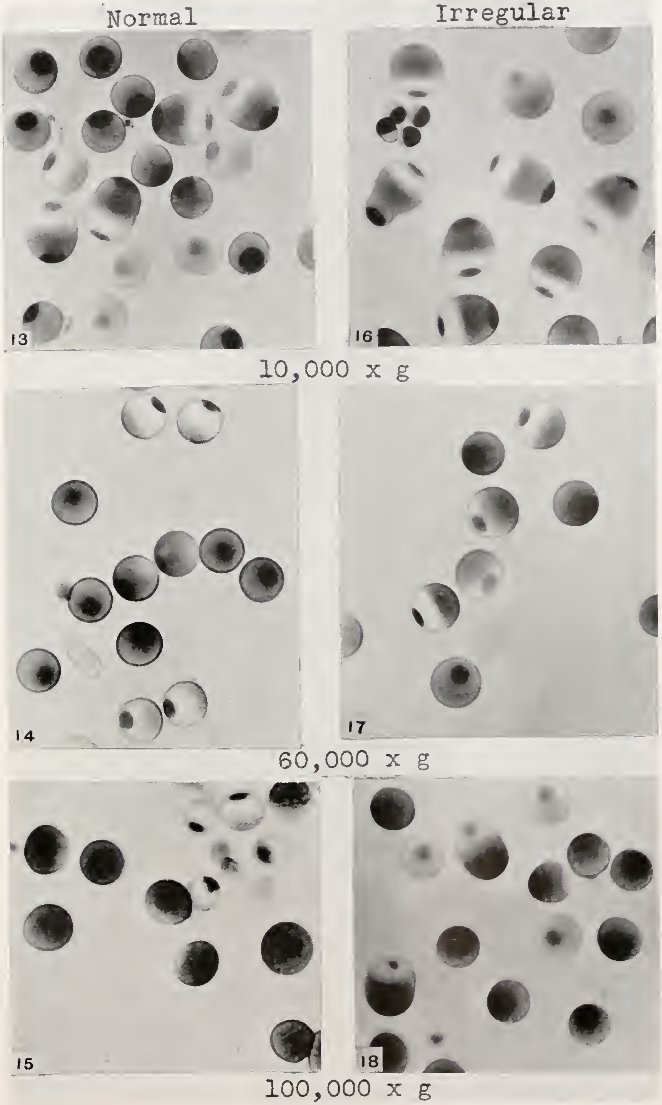
Normal
Irregular
13
16
10,000 x g
14
17
60,000 x g
15
18
100,000 x g

PLATE II

esculenta[2] (from Bermuda), the reverse relation was found, i.e. the greater the force, the larger the light half and the smaller the heavy half. In these eggs, the yolk granules are lighter than the matrix and lie under the oil, and the clear layer is formed more centrifugally; this must be the explanation of the apparent inconsistency in the relative size of the half-eggs of these two species. Whenever the granules are well segregated (low force) so that there is a large clear layer, the sphere with this layer is larger. Conversely, when the egg pulls apart before complete segregation of granules (high force), the clear layer is small, and the sphere containing this layer is smaller.[3]

The fact that one can vary at will the relative sizes of the two half-eggs and their composition by simply changing the centrifugal force gives a new tool for investigating the properties and functions of the different constituents of the egg. The principle may well be applied to the study of other marine eggs, and to other types of cells. For any accurate chemical work on the two half-eggs, the centrifugal force by which they are obtained must be given careful consideration. For complete segregation of materials, a low force should be used for a long period, even though one may actually break the egg in two subsequently with a high force.

Development

The small white fragments obtained with very high forces will develop when fertilized, if not too small. The smallest to develop were 32 μ in diameter, having a volume of only 7 per cent of the whole egg. Some of these formed skeletons though they did not become perfect plutei. The early cleavages of the small white fragments are quite regular.

The large red halves obtained with the high centrifugal forces cleave more regularly and develop very much better, both fertilized and parthenogenetic, than those previously obtained with lower forces. This is surprising in a way, because one might suppose that these tremendous forces would completely disorganize the cytoplasm. It has, however, been found by Beams and King (1936) that *Ascaris suum* eggs could be centrifuged at 400,000 × g for an hour in the 2- and 4-cell stage and 90 per cent of these would still continue to develop when removed; and they would develop also after being centrifuged at 150,000 × g for 4½ days. They would even cleave while being centrifuged at 100,000 × g. These eggs are, of course, protected from disruption by a very heavy shell. The red halves of *Arbacia* are also apparently not disorganized by these high forces, and they are almost as large as the whole egg (Photograph 15) and contain some of each kind of material present in the whole egg. Only the nucleus, a little of the oil and a little of the clear matrix are lacking. This is, then, a nice technique for practically separating the nucleus from the rest of the egg. So far, these non-nucleate halves, when activated artificially—the parthenogenetic merogones—have not developed further than the blastula. If such proves finally to be the case, we may conclude that eggs can cleave and pass through the early stages of development involving cell multiplication, without nuclei, but that for the later stages involving differentiation, nuclear material is necessary.

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[2] Data in original notes, 1932 (not published) for *Tripneustes esculenta:*

At 3,000 × g for ½ hr. Light half, D. = 76 μ; Vol. = 230,000 μ^3
Heavy half, D. = 51 μ; Vol. = 69,500 μ^3
At 7,000 × g for ¼ hr. Light half, D. = 79 μ; Vol. = 258,000 μ^3
Heavy half, D. = 43 μ; Vol. = 41,600 μ^3
The whole egg, D. = 83 μ; Vol. = 300,000 μ^3.

[3] Recent studies at Pacific Grove show that *Strongylocentrotus franciscanus* is stratified like *Parechinus* and *Tripneustes,* with the yolk granules in the light half; this half increases in size with increased force. In *Strongylocentrotus purpuratus,* two types of eggs occur, sometimes in the same batch, one type with the clear layer in the light half (like *Arbacia*), and the other type with the clear layer in the heavy half (like *S. franciscanus*). The heavy half becomes larger with increased force when it is granular, and the light half becomes larger with increased force when *it* is granular. There seems no doubt, therefore, that the distribution of the granules determines the size of the halves in relation to the centrifugal force; the more granular half becomes larger with a greater force.

SUMMARY

1. *Arbacia punctulata* eggs have been broken into "halves" with centrifugal forces ranging from 4,000 × g to 100,000 × g, the higher forces being obtained with the air turbine.

2. The relative size of the two halves varies with the centrifugal force used; the higher the force the larger is the (heavy) red half, and the smaller the (light) white half. With low forces, the white half is larger than the red; with high forces, the reverse holds.

3. The degree of stratification of the eggs just prior to breaking also varies with the force used. With low forces, applied for a long period (20 min.), the eggs are very well stratified. With high forces, the egg breaks apart (½–1 min.) before the materials are completely segregated into layers.

4. The red half obtained with the highest force available (100,000 × g), is only slightly smaller than the whole egg and contains some of all the materials in the original egg except the nucleus. It develops much better, both fertilized (fertilized merogone) and parthenogenetic (parthenogenetic merogone), than the red half obtained with lower forces previously used.

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EFFECTS OF ROENTGEN RADIATION ON THE JELLY OF THE ARBACIA EGG[1, 2]

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Freshly shed *Arbacia* eggs are enclosed by a layer of transparent jelly which can be easily demonstrated by adding a few drops of Janus green B (Harvey, 1939) to the water containing the eggs. When the Janus green is added, the jelly is first outlined, then is stained completely, eventually becomes brittle, and finally separates into long shreds which settle to the bottom of the container. The jelly is slowly lost, but when the eggs are kept in cool fresh sea water it can still be demonstrated after several hours. The jelly affords some mechanical protection to the egg, contains sperm agglutinin (Lillie, 1915; Just, 1930; and Tyler and Fox, 1939), and probably facilitates the preliminary stages of fertilization. Lillie (1915), Woodward (1918) and Tyler and Fox (1940) have shown that the agglutinin is non-dialyzable. Glaser (1914) and Woodward (1918) applied a number of common protein tests and obtained no reaction except that of weak xanthroproteic test. Tyler and Fox (1939, 1940) have strong evidence for the protein nature of the sperm agglutinin of the sea-urchin *Strongylocentrotus purpuratus*. These investigators also found that the jelly was dissolved by the proteinase, chymotrypsin. Tyler (1940) concluded that the agglutinin is either the jelly itself or a component of it. Although the above evidence is indirect as regards the jelly, it appears likely that it is protein in nature. The effect of Roentgen radiation on the jelly is so striking that we believe it is worthy of note even though little is known of the chemical nature of the jelly or of the mechanism involved in this phenomenon. We shall describe briefly the experiments from which the following results have been obtained. The radiation, under conditions described, removed the jelly from the eggs. Heavier dosages produced a change in the staining reaction of the *Arbacia* jelly which had been removed from the eggs by mechanical means.

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[1] Aided by a grant from the Rockefeller Foundation for Research on Physiology of the Cell.

[2] We wish to thank Drs. E. B. Harvey, J. H. Bodine, Gordon Marsh and Jay A. Smith for suggestions concerning the experiments and the manuscript. We also wish to thank Dr. E. P. Little for his kind coöperation in doing the irradiations.

Materials and Methods

Unless otherwise specified, the eggs employed were those of *Arbacia punctulata*, collected during June and July at Woods Hole. About 0.5 ml. of eggs in 10 ml. of solution was used for each sample and were irradiated in covered dishes made of Turtox plastic. Before and after the irradiation the eggs were kept in finger bowls at room temperature. For experiments involving jelly alone the eggs were allowed to stand for a time in sea water, were then shaken, centrifuged gently, and the supernatant fluid (containing the jelly) was poured off. The solution was irradiated immediately in the plastic dishes and then poured into Pyrex centrifuge tubes. Determinations of pH were done as soon as possible after the irradiation and were usually completed within an hour after the treatment. The pH measurements were made at room temperature (22–27° C.) with a Leeds and Northrup potentiometer-electrometer No. 7660, equipped with a small modified MacInnes glass electrode (MacInnes and Belcher, 1933). The electrode was calibrated with " standard acetate " for which the value pH 4.64 was taken (MacInnes et al, 1938).

The radiation characteristics were: 192,000 volts; 20 ma.; 6,100 roentgens per minute; target distance 9.5 cm.; filters consisting of glass walls of tubes and 5 mm. of bakelite (equivalent inherent filter equal to 0.2 mm. copper). Two tubes were used simultaneously. They were opposed, parallel and self-rectifying.

The buffers used were: acetate at pH 4.0, 4.4, 4.9 and 5.3; phosphate at pH 5.7 and 6.0; glycylglycine at pH 7.4, 9.0 and 9.4; and piperazine at pH 10.2.

Experimental Results

Removal of the Jelly from the Eggs by the Radiation

Relation between Dosage and Loss of Jelly.—The first experiments served to confirm the results reported in preliminary notes (Evans and Beams, 1939; and Evans, 1940). As the dosage of radiation was increased, the halo of jelly around the egg became progressively thinner until it was entirely gone. The relation between the dosage and number of eggs completely denuded of jelly is shown in Fig. 1, curve 1. The individual eggs varied as to amount of jelly present at the beginning of the experiments and this probably explains why some eggs were denuded by low dosages whereas others required heavier irradiation. Curve 2 of Fig. 1 shows the results of a similar experiment with the eggs of *Asterias forbesii,* and it can be seen that this jelly is more resistant than that of the *Arbacia* egg. The dosages required to remove the jelly from all of the *Arbacia* and *Asterias* eggs are high enough to produce abnormal development subsequent to fertilization. Roentgen radiation, therefore, is not suggested as a means of removing the jelly from eggs in which normal development is desired.

Is the Jelly Affected Indirectly by the Radiation?—A number of experiments have been performed in an effort to determine whether the effect on the jelly was produced directly by the radiation or indirectly through the action on some other substance in the medium. The following results seem to indicate that the effect was due directly to the radiation. (1) There was no apparent latent period. (2) The removal action was not prevented by low temperature (0° C.). (3) The jelly was removed by the radiation in solutions buffered at pH 7.4, 9.1, and 9.6. (4) Eggs were placed in heavily irradiated sea water and in water from eggs previously irradiated, and in both cases the jelly remained intact. (5) In two experiments female *Arbacia* were irradiated, and it was found that the eggs shed later were devoid of jelly.

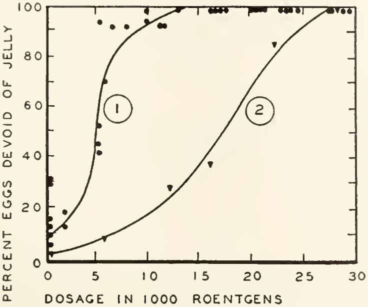

FIG. 1. A graph showing the relation between the dosage of radiation and the percentage of eggs devoid of jelly. Curve one is drawn through the values found in nine experiments on eggs of *Arbacia punctulata*. Curve two is drawn through the values found in one experiment on eggs of *Asterias forbesii.*

Action of the Radiation on the Jelly after its Removal from the Egg

At the suggestion of Dr. Robert Chambers, we attempted to study the effect of the radiation on the jelly after its removal from the egg. It was found if the jelly was removed from the eggs, that upon addition of a few drops of 2 per cent Janus green the jelly would react vigorously with the stain and would accumulate into one large clump at the surface of the water. As is shown in Table I, the dosages required to produce a negative test are greater than that necessary to remove the jelly from the eggs.

Effect of pH on the Jelly.—Inasmuch as acid sea water has been used to remove the jelly from the eggs (Harvey, 1939; Just, 1939; Tyler, 1940; Tyler and Fox, 1940), it seemed of interest to determine whether the radiation might increase the acidity of the egg medium enough to account for the removal of the jelly. One of the first steps in this investigation was to determine what pH range was effective in removing the jelly. It was found that at a pH of 4.9, or below, nearly all of the eggs lost their jelly within five minutes. Jelly alone was placed in solutions buffered at different pH values and it was found that at pH 4.0 the Janus green test was negative, and at pH 4.9 it was very faint, but at pH 6.0 to pH 10.2 the test was strongly positive. These results indicate that a marked change in the properties of the jelly is brought about by solutions whose pH values are as low as 4.0. This finding is in accord with that of Tyler (1940), who used solutions of pH 3.5 to dissolve the jelly.

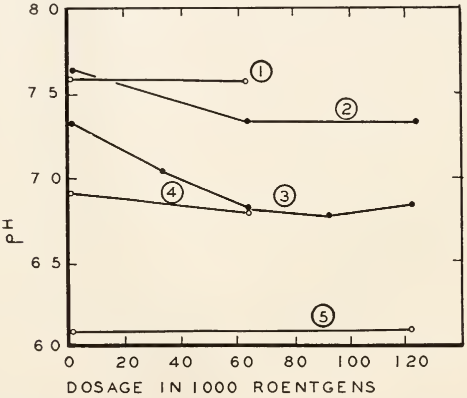

FIG. 2. Effects of Roentgen radiation on the pH of certain media. Curve one—jelly in medium buffered at pH 7.58. Curve two—jelly in sea water. Curve three—jelly in NaCl–KCl mixture. Curve four—eggs alone in the NaCl–KCl mixture. Curve five—supernatant fluid from cytolyzed eggs.

Effect of the Radiation on the pH of the Solution.—Sea water was irradiated and in two experiments where the dosage was 121,000 r the maximum lowering was only 0.06 of a pH unit. Fresh eggs placed in such irradiated water retained the jelly layer intact.

Irradiation of freshly-shed eggs in sea water apparently produced some acidity in the medium. In a representative experiment the pH was changed from 7.9 to 7.3 by an irradiation of 157,500 r. Conditions which were found to alter the pH change were: (1) amount of radiation, (2) length of time eggs had been in sea water before irradiation, (3) length of time eggs had been in sea water after the irradiation and before the determination of the pH, (4) mechanical injury to the eggs during the preparation of the supernatant fluid for pH determination, and (5) original concentration of eggs (and jelly).

Irradiation of eggs (with jelly) in unbuffered solutions of 19 parts 0.52 M NaCl to 1 part 0.53 M KCl produced a marked lowering of the pH. In a typical experiment the pH was lowered from 6.6 to 6.2 by an irradiation of 122,000 r.

It appears that irradiation of either eggs or jelly produces some acidity. As indicated in curve 3 of Fig. 2, when jelly alone was irradiated (61,000 r) in the NaCl–KCl mixture the pH was lowered about 0.4 units. The same dosage on jelly in sea water (curve 2) lowered the pH about 0.3 units. When eggs were separated from the jelly and irradiated in the NaCl–KCl solution (curve 4) again some acidity was produced in the supernatant fluid.

It is interesting to note, in connection with the question of the mechanism of the radiation action on the jelly, that a dosage sufficient to cause the negative Janus green reaction did not affect the pH of the buffered solution (curve 1). This indicates that a pH change is not necessary for the radiation action on the jelly.

Chambers and Pollak (1927), Pandit and Chambers (1932), and Krahl and Clowes (1938) agree that injury to the starfish and *Arbacia* egg lowers the pH which is usually changed from about 6.8 to 5.3. The present writers cytolyzed eggs by shaking them vigorously in various dilutions of sea water with distilled water and the lowest observed pH was 5.5. When the initial pH was this low, as shown in curve 5 of Fig. 2, the radiation produced no further shift toward the acid side.

The greatest change in pH produced by irradiation was obtained by adding the supernatant fluid from irradiated eggs (with jelly), in the NaCl–KCl solution, to a fresh lot of eggs and repeating the radiation treatment. In one experiment where the addition of fresh eggs and jelly was done twice, the pH was lowered by the radiation (total of 274,500 r) from 7.9 to 7.2. It is interesting to note here that the lowering of the pH by the radiation was not enough to remove the jelly from freshly-shed eggs. These findings are in agreement with those published in a preliminary report (Smith and Evans, 1940).

Effect of the Radiation on the Sperm-agglutination Property of the Jelly.—Richards and Woodward (1915) destroyed the sperm agglutinin of *Arbacia* egg-water by means of Roentgen radiation. We performed some experiments to determine whether the radiation would affect the sperm-agglutination property of the jelly in the same manner as the destruction of the ability to give the positive Janus green reaction. The results are shown in Table I, and it can be seen that the dosages of radiation required to destroy these two properties are somewhat similar. In a few instances it was possible to obtain a weak agglutination test where the Janus green reaction was negative. The response of these two properties of the jelly to the radiation are somewhat similar, yet their reaction to acidity is quite different. We have observed that an acid medium destroys the ability of the jelly to stain with Janus green, whereas Tyler (1940) has observed that the acidified egg-water is rich in agglutinin titer.

TABLE I

Exp. no.	Lot	Medium	Amt. of agglutination	Jelly
81	control	sea water	heavy	present
81	122,000 r	" "	none	none
84	control	" "	heavy	present
"	61,000 r	" "	light	absent
"	122,000 r	" "	none ?	absent
90	control	50% NaCl–KCl	heavy	present
"	30,500 r	" "	heavy	some present ?
"	61,000 r	" "	light	absent
"	91,500 r	" "	?	absent
"	122,000 r	" "	none ?	absent

DISCUSSION

Proteins in suspension are denatured and coagulated after long exposures to Roentgen radiation (Clark, 1936). The literature of radiation action on agglutinins has been reviewed by Brooks (1936) and he summarizes the suggested hypotheses as: (1) oxidation following primary activation of oxygen, (2) electrical discharge of colloid particles by alpha and beta rays, and (3) reduction processes. It is impossible at the present to give definite evidence for any mechanism of the action of the radiation on the *Arbacia* jelly, but the following observations may be suggestive. (1) The action is rapid. (2) It is not prevented by low temperature. (3) The increase in acidity is small. (4) The removal of the jelly from the egg, the loss of the Janus green reaction in solution, and the loss of the agglutination property appear to be three stages in the degree of separation of the jelly particles. In view of the observations noted above it may be that the radiation action involves a change in the charge on the jelly particles causing them to be repelled from each other. The action might also be expressed as an increased affinity for water. The mechanism might be the same as the action of Roentgen and radium radiation on the jelly of the *Nereis* egg. Packard (1915) irradiated this egg with beta rays of radium and observed an increase in the perivitelline space subsequent to fertilization. Redfield and Bright (1918, 1919, 1921) and Redfield, Bright, and Wertheimer (1924) exposed *Nereis* eggs to radium and Roentgen radiation and also observed an increase in the perivitelline space after fertilization. These authors were able to demonstrate a quantitative relationship between the radiation and amount of swelling. The intensity of the radiation and temperature were also found to be factors. The swelling of the jelly was attributed to the absorption of an abnormal amount of water. Costello and Young (1939) suggest that the radiation initiates the outflow of cortical jelly precursor and alters either the vitelline membrane or the jelly in such a way that the passage through the membrane is completely or partially prevented.

Summary and Conclusions

Roentgen radiation (6,100 r) removes the jelly from the *Arbacia* egg. When jelly is mechanically removed from the eggs it may be demonstrated in the supernatant fluid by its reaction with Janus green. An irradiation of 61,000 r will alter the jelly so that the Janus green test becomes negative. The jelly is not affected by solutions previously irradiated. The radiation produces a slight shift in the pH of unbuffered egg-water toward the acid side. The ability of the jelly-water to agglutinate sperm is greatly decreased by an irradiation of 61,000 r.

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GONOPODIAL CHARACTERISTICS PRODUCED IN THE ANAL FINS OF FEMALES OF GAMBUSIA AFFINIS AFFINIS BY TREATMENT WITH ETHINYL TESTOSTERONE [1]

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INTRODUCTION

The gonopodium of the male of poeciliid fishes is a highly modified anal fin used in the intromission of sperm. During juvenile stages the anal fins of males and of females are practically identical in structure but the fins of the two sexes become structurally divergent with the approach of maturity. Rays 3, 4 and 5 of the male fin undergo elongation and six differentiation areas arise within which characteristic hooks, spines, serrae and plates develop. In the female fin there is no marked elongation of the 3–4–5 ray complex and the differentiation areas do not arise to elaborate the morphological features peculiar to them.

Regnier (1938) was able to induce the development of some of the gonopodial characteristics of the male in the anal fins of the females of *Xiphophorus helleri* and *Lebistes reticulatus* by intramuscular injections of testosterone propionate, thus proving that the genetic factors for a gonopodium are borne by the female but that, in the absence of an androgenic hormone, they are not expressed. Grobstein (1940) obtained the same result in *Platypoecilus maculatus* but was able to demonstrate in addition that an anal fin of an adult female produced the features of the male gonopodium more exactly if that portion of the fin which gives rise to the male characteristics was cut away and allowed to regenerate during the treatment with testosterone propionate. Eversole (1939) was able to secure the formation of atypical gonopodia in females of *Lebistes reticulatus* at various stages by injecting testosterone propionate peritoneally and by adding testosterone propionate to the ration. The writer is indebted to Eversole for the information that pregneninolone (ethinyl testosterone) and other androgenic hormones will produce a similar effect.

The writer has studied in detail the normal development of the anal fins in males and females of *Gambusia affinis affinis* (Turner, 1941*a*) with particular attention directed to the origin of the marked differences between the mature fin of the female and the gonopodium of the male. In a second study (Turner, 1941*b*) fins or parts of fins were excised during development and allowed to regenerate. Some conclusions were drawn concerning the factors which control the normal development of the characteristic structures of the gonopodium. The discovery by Regnier, Grobstein and Eversole that the female anal fin could be made to develop into an atypical gonopodium has made it seem worth while to the writer to carry out similar experiments in *Gambusia affinis* with the objective of comparing in detail the development of the male features in the fins of treated females with those developed by males normally and under hormone treatment. From the results obtained in these experiments some conclusions can now be drawn concerning the effects of an androgenic hormone upon the development of the various structures of the gonopodium in females of *Gambusia affinis* which apparently possess genetic factors for the development of gonopodia but do not develop them in the absence of a hormone.

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In the experiments ethinyl testosterone [2] was added to the water of the aquaria in which the fishes were kept in the dosage indicated by Eversole (1.25 mg. of the hormone to 2000 cc. of water). All specimens were maintained at a temperature of 18° to 21° C. during the experiments.

NORMAL DEVELOPMENT OF FEMALE ANAL FIN AND GONOPODIUM OF MALE

The anal fins of males and females in early juvenile stages are practically identical in structure. As the female fin develops, all of the ten rays increase in length and segment regularly. A primary bifurcation of rays 4 to 9 occurs at the 10 mm. stage, a secondary bifurcation at approximately the 22 mm. stage and a tertiary bifurcation in rays 8 and 9 when a specimen has reached a length of 40 to 45 mm. Growth and segmentation of rays are continuous throughout the life of the specimen. There is no fusion of the branches of the rays. Some joints between the segments are obliterated by anchylosis beginning with a single joint in each segment at about the 8 mm. stage. The anchylosis proceeds with age until the basal parts of rays and about one-half of the primary branches are involved in old specimens 48 mm. in length. Rays 3, 4, 5 and 6 are longest and rays 5, 6 and 7 contain the largest number of segments.

The anal fin of the male differs from that of the female in late juvenile stages in the differential growth of the rays, in bifurcation of rays and in the degree of anchylosis of basal joints. Rays 3, 4 and 5 become greatly elongated during a period of rapid growth in which the growth of the other rays is subordinated. A single bifurcation takes place in all rays except 1, 2, 3 and 10 and the anchylosis of basal joints is very limited. During the latter part of the period of accelerated growth the differentiation areas appear (Fig. 1, *A*). Tissue lying along the ventral side of ray 3 thickens the ray (Area I). Area VI encompasses part of the branched portions of ray 5 and of rays 6, 7 and 8 at the same level.

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[2] A supply of the hormone was obtained through the kindness of Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey.

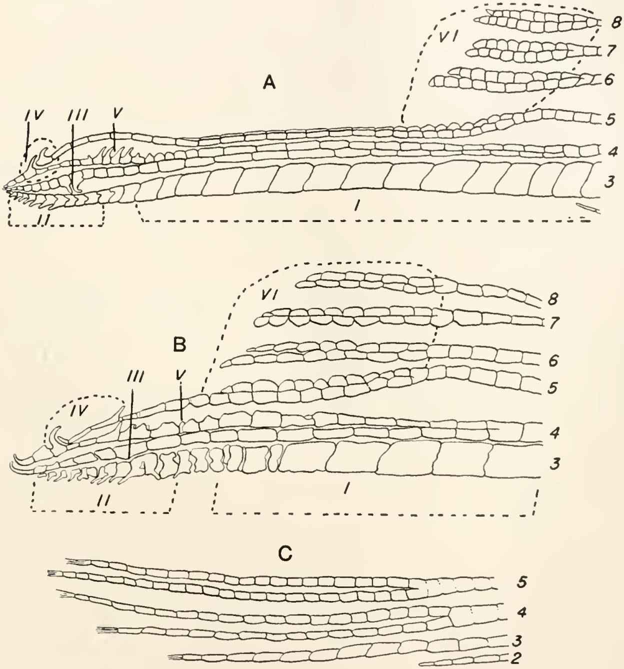

FIG. 1. Arabic numerals indicate fin rays numbered from the ventral side of the fin. Roman numerals indicate location of differentiation areas.

A. Rays 3 to 8 and differentiation areas in the anal fin of a normal mature male of *Gambusia affinis affinis*. The basal portions of the rays are not shown.

B. Rays 3 to 8 and differentiation areas in the anal fin of a 15 mm. female of *Gambusia affinis affinis* after 30 days of treatment with ethinyl testosterone.

C. Rays 2 to 5 in the anal fin of a normal 22 mm. female specimen of *Gambusia affinis affinis.*

Fusion of the branches and thickening of the segments of rays takes place in this area. Areas II, IV and V arise near the end of the differentiation period and Area III arises last. The hooks, spines, plates and serrae which have their origin in these areas are indicated in *A* of Fig. 1. Growth of the rays of the gonopod is concluded and terminalized when the specialized structures within the differentiation areas have been formed.

GONOPODIA OF TREATED MALES

Females exposed to treatment with ethinyl testosterone are obviously placed in a situation markedly different from that of males which are untreated, especially since the dosage of androgenic hormone used is much in excess of that necessary to produce the male characters. It is better to compare the gonopodia induced in females with those of males which have been subjected to the same treatment rather than with males which have been allowed to develop normally. For a first point of departure, therefore, a comparison is made between the gonopodia of normal untreated males and those of males treated during development.

TABLE I

Ray	Number of segments		
	Normal male specimen in 16-segment stage	Treated male at conclusion of gonopodial development	Normal untreated male at conclusion of gonopodial development
Ray 1	9	12	9
Ray 2	12	15	14
Ray 3	15	30	38–43
Ray 4	21	32	43
Ray 5	23	31	40
Ray 6	22	25	25
Ray 7	19	22	21
Ray 8	16	18	17
Ray 9	13	16	13
Ray 10	8	11	8

Males in the 16-segment stage (16 segments in the undivided third ray) will complete their gonopodial development and differentiation within a period of 35 to 60 days. The specific morphological features at the conclusion of development are shown in Fig. 1, *A*. Males in the same stage of development will complete their development in 30 to 40 days when treated with ethinyl testosterone.

Table I shows the segment number in each ray of the anal fin of normal specimens at the beginning of the experiment, of the treated specimens at the conclusion of the experiment and of fully mature untreated males. The outstanding difference between the fully developed gonopodia of normal and of treated males, as far as segment number is concerned, is in the 3–4–5 ray complex. The explanation of the difference lies in the relation of growth and segmentation in these rays to the formation of the differentiation areas. In normal gonopodia development of a low concentration of androgenic hormone from the testis induces accelerated growth and segmentation in the 3–4–5 ray complex. As the testis develops further and more hormone is liberated from the testis the developing fin responds by giving rise to differentiation areas and with the development of these, growth is terminalized. In the treated specimens the concentration of the hormone is so high that the development of the differentiation areas is quickly evoked and, although growth in the 3–4–5 ray complex is stimulated, it is soon curtailed. Rays 1 and 2 on the ventral side of the fin and rays 9 and 10 on the dorsal side are not involved in terminalizing differentiation areas and the growth-stimulating effect of the hormone becomes evident in slight increases in length and segment number in these rays as compared to the same rays in the normal gonopodium.

Some of the differentiation areas develop normally in the treated males while others do not. Area I in untreated males lies along the ventral side of ray 3 and adds new tissue to the segments as they are formed with the result that the rays are thickened. When Area II appears the effect upon ray 3 is to add a series of about eighteen new segments within 25 days. The new segments become progressively shorter and thinner and on the ventral side develop characteristic spines and plates. The transition in the character and size of segments from Area I to Area II is gradual. In the treated males there is an abrupt change in the length of segments in ray 3 between Area I and Area II. There is an indication that Area II has an immediate terminalizing effect upon further growth and regular segmentation of the ray, so that there are fewer segments in the ray when terminalized and there is no gradual transition in length of segments where Areas I and II overlap. The spines and plates which are developed on the ventral sides of the segments in Area II are fairly normal. Area VI in the treated male is almost identical with that of a normal untreated male. The appearance of Areas IV and V on rays 4 and 5 have the same effect of terminalizing growth and segmentation. From nine to eleven fewer segments are formed in rays 4 and 5 than in a normal gonopodium. Area IV, within which in the normal gonopodium a terminal hook develops on the dorsal branch of ray 4 and a second smaller, closely associated terminal hook on the ventral branch of ray 5, arises in the treated specimen in the usual position with reference to the ends of rays 4 and 5. However, the relative lengths of rays 4 and 5 are not quite normal, ray 5 being shorter. When the terminal hooks form on the two rays they are well separated in the treated specimen. Area V in normal untreated specimens is at first a condensed mass of tissue on the dorsal branch of ray 4 beginning about six segments from the end and extending basally for approximately eight segments. Within it arise vertical curved serrae which are laterally paired and placed one to each segment. Occasionally in the anterior segments of the series two low elevations may arise instead of one. In the treated specimen the development and final appearance of the structures of the area are normal except for a tendency to include a few additional segments at the anterior end. The relation of Area V to Area IV is constant with four segments intervening. Area III is the last to appear in both normal and treated specimens. It is likely to be lacking altogether in treated specimens or to be poorly developed.

When ethinyl testosterone is administered to males in juvenile stages before the 3–4–5 ray complex has begun to predominate in growth, the same result is obtained in the early evoking of the differentiation areas. However, growth in the 3–4–5 ray complex is stimulated by the same hormone and the earlier the stage treated, the greater the degree of growth and segmentation of the rays before terminalization. There will actually be a smaller number of segments in the 3–4–5 ray complex in a completely differentiated gonopod of a younger treated specimen, but the relative number of segments added to the rays after the beginning of the treatment will be greater. For example, a young normal male in the 8-segment stage will have 8 segments in ray 3, 11 segments in ray 4 and 12 segments in ray 5. Treatment with ethinyl testosterone induces the early formation of a gonopodium which will have, when complete, 25 segments in ray 3, 25 segments in ray 4 and 26 segments in ray 5. Ray 3 will have added 17 segments and rays 4 and 5 will have added 14 segments each. The treated specimen already described (at the 16-segment stage) will have added only 15 segments to ray 3, 11 segments to ray 4 and 8 segments to ray 5.

The effect of treating males during the development of the gonopodium with large dosages of ethinyl testosterone are: (1) to evoke prematurely the formation of the differentiation areas. (2) To terminalize prematurely growth in the 3–4–5 ray complex. Growth in these rays is normally terminalized when differentiation areas appear but the early induction of the differentiation prematurely terminalizes growth and differentiation. (3) To produce secondarily some abnormal conditions in the differentiation areas themselves. The normal formation of an area which involves parts of two rays (Area IV) depends upon the attainment of a specific growth stage in each ray by the time differentiation occurs. If treatment with hormone is started at a stage before one of the rays has attained the usual growth stage the differentiated structure may be abnormal. (4) To stimulate growth and segmentation in the 3–4–5 ray complex if the hormone is administered before these rays have entered their accelerated growth period.

GONOPODIAL STRUCTURES INDUCED IN THE ANAL FINS OF FEMALES

The extent to which an anal fin of a female can be modified during its development depends upon the extent to which the fin has become structurally fixed in the female pattern. Modification by treatment with ethinyl testosterone does not produce a regression of any structure already formed. Addition of new tissue to old structures or renewed or accelerated growth of structures already present are the mechanisms of modification. A closer approximation to the pattern of the mature gonopodium of the male may be induced more readily in a young female specimen, where a mature pattern of neither male nor female has been laid down, than in an old female where growth, segmentation, additional bifurcation and anchylosis of basal joints of rays have fixed the structure of the fin in the adult female pattern.

Female Fifteen mm. in Length

In the anal fin of a normal female at this stage rays 4 and 5 have bifurcated once and there is some anchylosis of the basal segments of the rays. Rays 5 and 6 are the longest and have the largest number of segments.

After 23 days of treatment with ethinyl testosterone rays 3, 4 and 5 have elongated and segmented so as to form a definite lobe and all the differentiation areas except Area III have made their appearance. At the end of 31 days differentiation is complete. An untreated male of this size and stage of development would have required about 45 days for the complete development of a gonopodium. At 23 days ray 3 added 11 segments, ray 4 added 13 segments and ray 5 added 6 segments. Differentiation of areas occurred prematurely and the growth of the 3–4–5 ray complex was terminalized sooner than would have been the case in a normal untreated male. In the complete gonopod of the treated female rays 3, 4 and 5 had 24, 29 and 24 segments respectively while an untreated male would have had 41, 43 and 42 segments in rays 3, 4 and 5 respectively.

All differentiation areas develop in normal positions with respect to the ends of rays 3 to 8 (Fig. 1, *B*). As a result of the early development of the fin in a female animal and the development of the terminal phases under the influence of the androgenic hormone, the base, which is unchanged, is that of a typical female while the terminal portion is, with some modifications, that of a male. At this stage there is little difference between the anal fins of males and females both of which have been treated with the hormone. In the female specimen the elbow of the ventral branch of ray 4 (Area III) is not so well developed and in Area V the vertical serrae are lower and the anterior segments of the gonopodium are inclined to have two pairs of serrae instead of the one occurring in males. An interesting variation of Area IV suggests that the synchronization of the end of the growth period with the onset of the differentiation areas which operates smoothly in the normal specimen is poorly integrated here. Some days in advance of the final stage of differentiation there appeared in the ventral branch of ray 5 what seemed to be the terminal hook. However, after the hook was formed the ray continued to grow and to segment (Fig. 1, *B*) until the terminal segment came into contact with the recurved terminal hook at the end of the dorsal branch of ray 4. In this position it formed a second smaller hook. Both branches of ray 4 also continued to grow a little beyond the position of the usual terminal hook and, after adding a long segment, formed small but definite second terminal hooks. Apparently, here the axial growth of the rays, which is terminalized in normal gonopodial development with the differentiation of the terminal hooks, was not terminalized completely and continued on so as to add new segments beyond the differentiated areas.

Females Twenty to Twenty-eight mm. in Length

Anal fins of females at this stage of development are rounded in outline with the central rays the longest. The unbifurcated bases of rays 4 to 9 are partially or wholly solidified by the anchylosis of the joints. Secondary bifurcations may occur in rays 7 and 8.

In specimens treated with ethinyl testosterone at this stage the 3–4–5 ray complex is visibly elongated within 18 days and within 31 days differentiation of the fin is complete. Ray 3 adds 13 short segments during this time and rays 4 and 5 add 7 and 8 segments respectively to the ends of the branches.

Figure 1, *C* represents the terminal parts of rays 2, 3, 4 and 5 of a normal female fin at this stage prior to treatment. Figure 2, *D* indicates the changes that take place within 28 days and Fig. 2, *E* is an enlarged camera drawing of the terminal parts of rays 3, 4 and 5 of another specimen in the same stage of development. Area II contains a smaller number of segments than in the normal male but the ventral spines, while atypical, are clearly like those in Area II in normal gonopodia. The elbow of Area III is hardly developed. Area IV, containing the terminal hooks of rays 4 and 5, is well represented but the terminal hook of ray 5 is poorly formed and is well separated from that on ray 4. Area V with its vertical serrae is developed in the same position with reference to the end of ray 4 and to about the same extent as in a treated 15 mm. female. Area VI is not as well developed as in the 15 mm. specimen. The basal portions of the rays with their

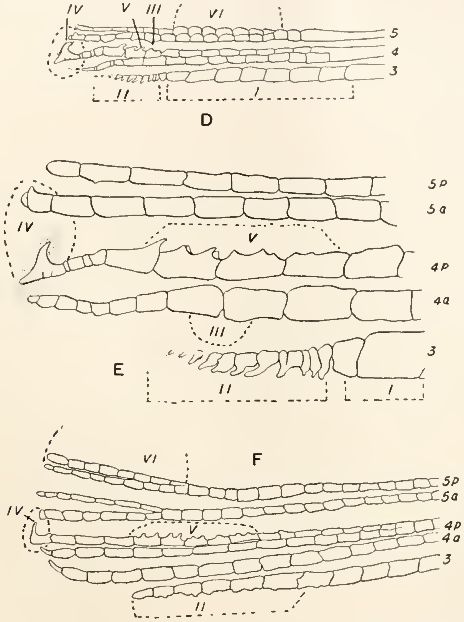

Fig. 2. *D*. Rays 3 to 5 and the differentiation areas in the anal fin of a 20 mm. female of *Gambusia affinis affinis* after 28 days of treatment with ethinyl testosterone.

E. Enlarged drawing of terminal parts of rays 3 to 5 of the anal fin of a 22 mm. female of *Gambusia affinis affinis* after 28 days of treatment with ethinyl testosterone. Differentiation of the fin is not quite complete.

F. Rays 3 to 5 and differentiation areas in the anal fin of a 38 mm. female of *Gambusia affinis affinis* after treatment with ethinyl testosterone for 36 days.

anchylosis were laid down while the specimen was developing as a normal female and no change in this portion of the fin is produced by the introduction of the hormone.

In general, the transformation of the fin at this stage is specifically male-like in the elongation of rays and the addition of segments to the 3–4–5 ray complex and in the development of specific differentiation areas characteristic of the male fin. In none of the differentiation areas at this stage is the transformation as completely male-like as in the specimen treated with ethinyl testosterone at an earlier stage.

Females Thirty-five to Thirty-nine mm. in Length

At this stage the anal fin of the female is rather definitely fixed in its structural pattern. It is still growing slowly and is adding new segments to the rays. The anchylosis of segments has proceeded apically from the base of each ray so that the unbifurcated basal portion and a considerable part of the secondary rami of the divided rays are solidified. Secondary bifurcation has taken place in the dorsal branch of ray 4 and in both branches of ray 5 with the result that there are now three termini in ray 4 and four in ray 5.

Treatment of specimens with ethinyl testosterone results in a specific but incomplete development of the differentiation areas of the gonopod but the addition of new segments is extremely limited. From one to four new terminal segments may be added to ray 3, a very small number as compared to the number that is added to ray 3 in the younger stage, but it is important that the specific response occurs even slightly. The terminal branches of rays 4 and 5 may add one or two short segments. It would appear that the capacity to respond to hormone stimulation by growth had nearly reached its limit at this stage.

The formation of the differentiation areas is also specific as in the anal fins of the treated females at younger stages but the development of the areas is less complete. Fig. 2, *F* represents the terminal parts of rays 3, 4 and 5 in the anal fin of a 38 mm. female which has been treated for 36 days. Area II is represented by a slight roughening on the ventral side of the last seven segments of ray 3. Area III in this specimen does not appear but in some specimens there is a slight thickening of two or three segments in the correct position on the ventral member of the dorsal branch of ray 4. Area IV is represented by a strong terminal hook on the dorsal member of the dorsal branch of ray 4. The terminal hook on the branch of ray 5 nearest to ray 4 is sometimes present but is always well separated from that of ray 4. The vertical serrae which form Area V are present in some specimens but are usually low. They are developed upon the usual number of segments (5 to 7) and the area is in the usual position beginning about four segments from the end. Area VI is poorly represented. In some instances there is a slight thickening of the dorsal members of ray 5 or there may be some fusion of the terminal branches but the modification does not extend to rays 6, 7 and 8.

Modification of the anal fin in females at this stage is much more limited than in younger specimens and consists of the addition of a few segments to rays 3, 4 and 5 and of a specific but incomplete development of the differentiation areas.

Discussion and Summary

The structural modification of the anal fin in a female treated with ethinyl testosterone follows the same course of development as that of the development of the normal gonopodium of the male, as far as possible, considering that the anal fin is already fixed to some extent in the female pattern and that the ethinyl testosterone is administered in a high dosage. In the development of the normal gonopodium a small amount of androgenic hormone, released from the testis, produces the initial effect of accelerated growth in the 3–4–5 ray complex. At the same time two of the six differentiation areas are induced to arise. Later, a larger amount of hormone is released from the testis and four other differentiation areas make their appearance. With the origin of these four differentiation areas growth and segmentation in the 3–4–5 ray complex is terminalized. When ethinyl testosterone in the dosage used here is introduced, the period of accelerated growth is induced, but before the 3–4–5 ray complex has become elongated to the normal extent, the second phase of development, terminalizing differentiation, is induced and growth is curtailed.

A specific pattern in the normal gonopodium is dependent upon a normal growth in the various rays at the time differentiation occurs. In the treated specimens, lacking the full extent of growth in each of the rays concerned, there are likely to be abnormalities in the differentiation areas themselves, particularly in Area IV where the two terminal hooks found in the area become spatially separated.

If the hormone is administered to a juvenile female the amount of growth in the 3–4–5 ray complex is greater and the resemblance of the fin in general and detail is nearer to that of the normal mature male than is the case if the hormone is administered to a specimen in which the fin is in a later stage of development. In old females there is practically no growth and the extent to which differentiation areas are formed is extremely limited. The differences in transformation at the different stages are due to two conditions: (1) The rate of growth of the fin becomes diminished in older specimens, presumably because of the onset of a slower metabolic rate in the tissue. It has been shown by the writer in regeneration experiments (Turner, 1941*b*) that capacity to regenerate diminishes in old specimens and that after the formation of the differentiation areas this capacity is lost entirely in those rays which are involved in the differentiation areas. In the same study it is shown that capacity to form new differentiation areas after they have been excised diminishes and disappears entirely once the areas have developed fully. Grobstein's experiments, in which it is demonstrated that, in older females, the formation of the differentiation pattern is more complete in regenerated than in normal senescent tissue of the anal fins of older females, indicate that tissue with a higher metabolic rate is more susceptible to transformation. (2) Since there is no dedifferentiation nor any absorption of tissue already formed in the transformation of the female fin, the development of the normal anal fin in the direction of the female pattern becomes a definite deterrant to transformation. Changes occur only by growth and segmentation of rays already formed and by the addition of new structures within the differentiation areas to the old or new segments. In old specimens, since there is little capacity to respond to hormone stimulation by growth, the modification of the fin is limited to such changes as can be accomplished by the addition of new structures to the framework of segments already elaborated. Furthermore, the reduced capacity in old specimens of the differentiation areas themselves to form the characteristic structures reduces the degree of change.

In general, it may be stated that the capacity for a female anal fin to become modified in the direction of the typical gonopod of a male is high in juvenile specimens, less in specimens somewhat older, and very limited in older specimens because of a fixation of the fin in a female pattern and because of a diminished capacity for growth and differentiation in older specimens. In older specimens the fin will be female in pattern but there will be additional structures formed within the six differentiation areas to the extent to which it is possible in a fin already fixed in the female pattern.

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MATING TYPES IN DIVERSE RACES OF PARAMECIUM CAUDATUM

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Introduction

Investigations on *Paramecium aurelia* (Sonneborn, 1937, 1938 *a* and *b*), and on *P. bursaria* (Jennings, 1938 *a* and *b*, 1939 *a* and *b*) have recently shown that these species consist of a number of mating types. As a rule, and possibly always, individuals of the same mating type will not conjugate with each other; but when cultures of certain diverse mating types are mixed together there follows under appropriate conditions an immediate agglutinative reaction leading to conjugation between animals of diverse types. These phenomena are of interest in themselves, and in relation to sexuality and self-sterility; and they provide a means by which the genetics of these organisms may be rapidly developed. It therefore appears desirable to investigate from this point of view a large number of diverse species so that there may be available a broad comparative body of knowledge of these phenomena.

For this purpose, *Paramecium caudatum,* a species not hitherto studied from this point of view, was selected for intensive investigation. This species was chosen because it is one of the commonest and most intensively investigated species of Paramecium and because its nucleus and chromosomes are moderately favorable for the cytological work that must eventually become correlated with the genetic analysis.

Four major problems have been attacked experimentally. The first and basic problem is the occurrence, interrelation and geographical distribution of the mating types. The second problem, for which no final answer is available, is the inheritance of mating type during vegetative reproduction. The third is the influence of various environmental factors (nutrition, time of day, and temperature) on conjugation following mixture of different mating types. The final problem was to discover, if possible, morphological or physiological differences between the diverse groups that could be distinguished by their breeding behavior.

Materials

The material used in the present work was derived from collections of *Paramecium caudatum* obtained from twenty-six natural sources in

Canada and in the states of California, Connecticut, Georgia, Kansas, Maryland, Massachusetts, and Pennsylvania. Soon after each collection reached the laboratory, one or more individuals were isolated and from each individual a large stock culture was developed. These ninety-three stock cultures were the ones employed in all the following experimental work. All the clones used were identified as *P. caudatum* by examination of temporary aceto-carmine preparations or, in a few cases, of permanent Feulgen preparations to determine the number and type of micronuclei.

I am indebted to the following people for supplying me with collections of *Paramecium caudatum:* Dr. T. T. Chen, Dr. Harold Finley, Father J. A. Frisch, S. J., Dr. A. C. Giese, Mrs. R. W. Gilman, Mr. C. B. Metz, Dr. T. M. Sonneborn, Mr. Samuel Steinberg, Dr. Vance Tartar, and Prof. D. H. Wenrich.

METHODS

The basic culture fluid, a lettuce infusion medium, was prepared as described by Sonneborn (1936), save that .75 grams of dried lettuce per liter was used instead of 1.5 grams. This fluid was lightly inoculated before use with a single unidentified species of bacteria grown on agar slants. This bacterium was isolated in the early stages of the work from a thriving culture of the paramecia.

The paramecia were cultured either as isolation lines on depression slides with daily transfer of single animals or as mass cultures in glass caster dishes with periodical transfer of a number of the animals to a fresh dish. In some cases, the mass cultures were fed by adding a grain of pearl barley, or a small piece of coagulated egg yolk to induce bacterial growth.

No effort was made to maintain absolutely sterile conditions but precautions were taken to insure the predominance of the desired bacterium in the culture. The glassware was sterilized by boiling or autoclaving, and the cultures were exposed to the air only long enough to allow the removal of animals for transfer or for experimental purposes.

At times, heavy bacterial growths (presumably of a contaminating bacterium) caused the appearance of heavy clouds of bacteria in the bottom of the slides or caster dishes. At other times, some of the cultures became contaminated by a small flagellate. The cultures were effectively purified of the contaminating organisms by running single animals in isolation lines for four days in succession. In making the transfers, the mirror of the microscope was tilted so that no light fell on the objectives and the contaminating organisms appeared as luminous dots. In this way it was possible to draw back up into the pipette most of the contaminating organisms transferred with the paramecium. This method reduces considerably the risk of injury to the animals which the repeated transfers used in washing by the method of Parpart (1928) involve. Although the method described merely insures a predominance of the desired bacterium, it was found to be entirely satisfactory.

Testing Cultures for Mating Types

The fundamental observation on which the concept of mating types is based is simply this: certain cultures in which conjugation does not occur when separate, conjugate when mixed together. Two such cultures that do not conjugate alone but do conjugate when mixed are said to be of different mating types. In order to ascertain whether there occur in *P. caudatum* mating types such as those found in *P. aurelia, P. bursaria* and other species of Paramecium, it was necessary to obtain cultures within which conjugation did not occur, to mix representatives of these in all possible combinations of two, and to observe whether conjugation occurred in the mixtures or not.

The cultures to be tested for mating types were the 93 from the various collections mentioned in the section "Materials." Previous work by Sonneborn (1938*a*) and Jennings (1938*a*) on other species of Paramecium has shown that it is unnecessary to make all possible combinations of two among the cultures examined, for they found that all cultures of the same mating type behave alike when mixed with any other culture. Therefore, in the present work, after mating types had been discovered, only one representative culture of each mating type was used for mixture with new cultures of unknown mating type. The rule, therefore, was to mix every unknown culture with every other unknown culture and with representative cultures of each known mating type.

Occurrence, Number, and Interaction of the Mating Types

As a result of mixing the various clones it was found that certain mixtures regularly gave conjugation while others regularly gave no conjugation. It was concluded, therefore, that mating types were present in *P. caudatum*. This agrees with the findings of Giese and Arkoosh (1939) who reported the presence of two mating types in *P. caudatum*. When the results were collected, it was found that the clones studied could be divided into at least four and probably five groups of two mating types each. The groups were numbered one to five in order of their discovery. The mating types of Group 1 were designated I and II, those of Group 2, III and IV, those of Group 3, V and VI, those of Group 4, VII and VIII, and those of Group 5, IX and X. Although this is the same nomenclature used by Sonneborn for *P. aurelia,* it implies no connection between the corresponding groups and types in the two species.

The interaction of groups and types in *P. caudatum* is shown in Table I. Each mating type in a group conjugates only with the other mating type in the group. Thus type I conjugates only with type II and not with other clones of type I or with clones of types III, IV, V, and VI, and so on for the other groups.

TABLE I

The relations among types and groups in *P. caudatum.* Conjugation is represented by a plus, absence of conjugation by a minus. A blank indicates that no mixture was made when both of the groups involved were known to be in reactive condition.

Group		1		2		3		4		5	
	Type	I	II	III	IV	V	VI	VII	VIII	IX	X
1	I	−	+	−	−	−	−				
	II	+	−	−	−	−	−				
2	III	−	−	−	+	−	−	−	−	−	−
	IV	−	−	+	−	−	−	−	−	−	−
3	V	−	−	−	−	−	+	−	−	−	−
	VI	−	−	−	−	+	−	−	−	−	−
4	VII			−	−	−	−	−	+	−	−
	VIII			−	−	−	−	+	−	−	−
5	IX			−	−	−	−	−	−	−	+
	X			−	−	−	−	−	−	+	−

It is not certain as yet that Group 1 is separate from both Group 4 and Group 5, since so far Group 1 has not been in condition to conjugate at the same time as Groups 4 and 5. However, from other considerations, it appears highly probable that there really are five groups of mating types. There remain, however, two collections from which no clones have so far conjugated when mixed with each other or with any of the groups of mating types. It is possible that these two collections may represent one mating type of a sixth group.

If samples of the two mating types in a group are mixed when in the proper physiological condition, there follows immediately the pronounced agglutinative mating reaction described by Sonneborn (1937) for *P. aurelia* and by Jennings (1939*a*) for *P. bursaria.* When the animals are put together they stick to those of the opposite mating type with which they chance to come in contact and form clumps which later break down into pairs which complete conjugation.

In regard to the geographical distribution of the various groups, it is to be noticed that Group 1 was found in two collections from Baltimore, Maryland, but not in collections from other localities. Group 2

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was found in six collections from Baltimore; one collection from Woodstock, Md.; one collection from New Haven, Conn.; one collection from Stanford, California; and two collections from Falmouth, Mass. Group 3 was found in three collections from Baltimore, three from Baldwin City, Kansas, one collection from Atlanta, Georgia, and one collection from an unknown locality in Connecticut. Group 4 was found in one collection from Baltimore, one collection from Waterville, Conn., and one collection from New Haven, Conn. Group 5 was found in a collection from Hamden, Conn. The two collections whose group is still undetermined were from Philadelphia, Pa. and Canada near Buffalo, N. Y.

In general, it can be said that there is no definite evidence of geographically isolated non-interconjugating groups of mating types. It is true that Groups 1 and 5 have been found in only one general locality, but since there is little material available for these groups this cannot be considered significant. Animals of the other groups have been found in widely separate localities.

Conjugation Within a Clone

There are some apparent exceptions to the rule that any clone in a group conjugates with only one of the two mating types in the group. However, in all these apparent exceptions, conjugation has also occurred in one of the control cultures, so that it is not a question of a clone conjugating with clones of the two mating types of a group but rather a question of conjugation between members of the same clone. As an example, some results of mixtures of five clones of type IV (A2 to A6) with a type III clone (S1) and a type IV clone (A1) will be given. In the mixtures with the type III clone, from 61 to 72 pairs were formed with from 7 to 11 pairs of conjugants in the mixtures with the type IV clone. However, no conjugants were found in the type III (S1) control, while ten pairs were present in the type IV (A1) control. Some conjugants were found in the A2 to A6 controls, ranging from none in A6 to eleven in A4. Thus it is seen that the conjugation in the mixtures with A1 was the result of conjugation *within* the various clones and not the result of mixture.

In the clones in which conjugation has been observed to occur in the controls set up when making mixtures, conjugation has also been observed in the source cultures. Many of the cultures have conjugated at times without mixture. There is a great deal of variation with respect to this phenomenon among different clones. In some clones there have been large percentages of conjugants at intervals of two weeks to a month. Each time the conjugation occurred in a culture which had been started from a single non-conjugant animal at the time of the previous occurrence of conjugation. The proportion of animals conjugating varied in these cultures from about 20 per cent to nearly 100 per cent. Other clones have conjugated at only one time during a period of nine to twelve months, and in these the proportion of animals conjugating varied from 5 per cent to 10 per cent. In still other clones, no conjugation has ever been observed except upon mixture with another clone of the proper mating type. Examples of the kind of clone in which large numbers of conjugants occur at short intervals have been found in both Groups 1 and 2. In Group 1, all three of the type II clones so far discovered are of this kind while in Group 2 two of the 7 type III clones and five of the 12 type IV clones are of this kind. Clones of the second kind in which small proportions of conjugants occur at long intervals have been found in two groups. In Group 1, there are four examples all belonging to type I, while in Group 3, two type V clones and two type VI cones are of this kind. In each of these, only a small proportion of the animals in one culture have conjugated at one time during the entire period of eight months to a year and a half that they have been under observation. Examples of clones which have never, while under observation, conjugated without mixture are found in all three groups: in Group 1, one type I clone; in Group 2, two type IV clones and one type III clone; and in Group 3, one type V and one type VI clone. The length of time that this condition has held true differs with different clones since some have been kept in the laboratory for longer periods than others. Four of the clones have been under observation for a year to a year and a half, and two for eight months.

Since conjugation does occur in this manner within a clone, the problem presents itself of whether or not two mating types have been produced within the clones as Sonneborn (1937) found in *P. aurelia* at autogamy. In order to test this matter, it was necessary to do two things: first to see if any autogamy or endomixis occurred (endomixis in *P. caudatum* has been reported by Erdmann and Woodruff, 1916, and by Chejfec, 1930), and if so, to see if conjugation in a culture without mixture depended upon its prior occurrence in the culture; second, to find out whether or not both of the mating types in a group were produced in a culture originally of one mating type and whether or not the differentiation into two types occurred at autogamy or endomixis.

In order to test the possibilities just mentioned, it was necessary to attack the problem in two ways. In the first place, to find if autogamy or endomixis were occurring, it was necessary to stain cultures daily with aceto-carmine. In pursuance of this plan, twenty-four clones were run in daily isolation lines with daily staining for a period of thirty days. During this time one-fourth to one-half of the animals in each of the depressions were removed each day, stained with aceto-carmine and examined with the compound microscope for nuclear changes indicative of autogamy. Although examples of clones which had, in caster cultures, been producing conjugants at intervals of from ten days to two weeks were included, no evidence of any nuclear changes was observed during the month the cultures were under observation.

Although the results indicated that conjugation was occurring in these cultures without previous autogamy, it was felt, since the tests for autogamy were made under isolation line conditions in depression slides and the conjugation which had occurred had been under mass culture conditions, that the environmental conditions in the two situations were sufficiently different so that autogamy might have occurred in the parent caster dish cultures even though none occurred in the isolation lines derived from them. It was therefore decided to stain representative samples from a caster dish culture that was originally derived from one animal of clone D4, a clone in which conjugation occurred frequently. This animal was allowed to multiply in a depression slide. As soon as a sufficient number of animals was present, they were transferred to a caster dish and sixty drops of culture fluid added. As soon as several hundred animals were present in the dish, a grain of pearl barley was added to give a constant supply of food, since it was under these conditions that conjugation was previously observed to occur when the paramecia in the isolation lines showed no indication of autogamy.

The procedure used in testing for autogamy was to stain a sample from the culture every day and examine for nuclear changes. When only a relatively small number of individuals was present in the culture one-fourth were stained with aceto-carmine for the daily examination until at least ten were being stained. From that time onward from ten to one hundred animals were examined daily. No evidence of nuclear reorganization was ever observed in this culture but practically 100 per cent of the animals were conjugating twelve days after the start of the experiment.

These results indicate (for clone D4 at any rate) that the conjugation observed is not the result of a previously occurring autogamy. There remained to be answered, however, the question of whether or not both mating types were present in a clone when conjugation occurred. In order to answer this question, pairs of conjugants which were not yet firmly united were separated by squirting them violently from a small bore pipette. The cultures derived from the animals separated in this way are known as split-pair cultures. Because of the difficulty of finding pairs which are not yet firmly united in cultures in which only a small proportion of the animals conjugated at one time during the course of the experiment, all the work with split pairs was carried on with those cultures in which large proportions of the animals conjugated at relatively short intervals. In Group 1, type II, clones D4 and C2 were used mainly with some additional work on clone D2. In clones D4 and C2, a number of pairs (17 in D4 and 18 in C2) were split and cultures grown from each member of the pair. Besides the split-pair cultures, a number of cultures were started from single non-conjugant animals from cultures in which conjugation was occurring. In Group 2, clone P (type III), twelve pairs of conjugants were split and cultures were grown from them. The clones were tested for mating type as soon as the population in the cultures had reached a sufficient density to make tests for type possible. However, conjugation again occurred in many of the cultures before it was possible to make the tests. The cultures which were tested were always of the same type as the parent culture. Among the split pairs from type II cultures, cultures from both members were tested and reacted in the case of two split pairs from D4 and one split pair from C2. Both members of each pair were type II, the same as the original culture. In the split-pair cultures from clone P both members of four pairs reacted as type III the same as the original culture. In addition, those split pairs in which only one of the resulting cultures gave a reaction and those cultures derived from isolated non-conjugating animals behaved as the same type as the parent cultures when tested for mating type.

In summary, it can be said that the results of the work on two clones in Group 1 (C2 and D4, type II) and one clone in Group 2 (P, type III) indicate that there is no permanent change of type when conjugation occurs within a culture since, whenever cultures from both members of a pair have given a test for mating type, both have been of the same type as the parent culture. Furthermore, the parent cultures still react as the same mating type they were when first isolated, even though they have been subcultured many times during the year to a year and a half that they have been in the laboratory.

These results do not allow any definite conclusion about possible temporary changes of mating type during vegetative reproduction such as Kimball (1939) found in *P. aurelia*. If such changes occur, they are temporary and the animals quickly revert to the mating type characteristic of the clone. The possibility that conjugation in these cases is between animals of the same mating type cannot be ruled out but seems unlikely in view of the previous work on Paramecium.

Although the results presented indicate that conjugation within a clone (selfing) is not the result of a change of mating type following autogamy, there are several points of interest in connection with such selfings. They occur only in cultures which have been in the same culture dish for a period of time—two weeks approximately if a grain of pearl barley is added to the culture, longer if the cultures are fed by the addition of lettuce infusion. If animals from such selfing cultures are removed during the period before selfing occurs and mixed with the appropriate mating type conjugation will take place, however, and continues to occur even when selfing has commenced in the culture. It can thus be seen that being in the proper condition to conjugate when mixed is not enough to induce selfing and that some additional factor is involved. Successive selfings can be readily obtained at approximately two-week intervals if cultures are started from single non-conjugant or split-pair animals and maintained under the conditions described.

There is one case of conjugation within a clone (Gilman, 1939) in which the situation seems to have been different from that so far reported. In this clone (M, Group 2, type III) when conjugation was observed the first time in an unmixed culture, four conjugant pairs were split before they had gone through conjugation and the clones derived from the two members were tested for mating type. In all four, one member gave rise to a type III and one to a type IV clone. Since this phenomenon did not recur, it is impossible to tell whether it was due to a change of mating type as a result of autogamy or some other nuclear change or whether it resulted from an accidental contamination of the original type III culture by type IV animals from another source.

The subsequent histories of the two types isolated from this culture were very different. The four type IV clones continued to react as type IV and no conjugation occurred in them without mixture during the year they were kept under observation. The four type III clones, however, contained conjugants again approximately six weeks after the pairs were split. New split pairs were obtained from these type III clones. The clones derived from the two members of these pairs were all type III. It appears, then, that conjugation within a clone may be the result of the production of animals permanently of both types as in clone M at the time of its first spontaneous conjugation or it may occur without the production of clones permanently of two types as in the subsequent conjugation in clone M and also in clones C2, D2, D4 (type II) and P (type III).

Conditions Necessary for Conjugation

Nutrition

Some observations of considerable interest have been made on the influence of the nutritive state on the clumping or mating reaction and on the subsequent conjugation. No detailed observations have been made with respect to this problem in Groups 3, 4 and 5. A little has been done with Group 1, but since the results obtained were essentially similar to those obtained with Group 2 and since more detailed observations were made with Group 2, only this group will be considered. In Group 2 five stages in nutritive decline associated with characteristic changes in the mating reaction have been observed. When cultures of types III and IV in these diverse conditions are mixed the following immediate behavior is observed:

(1) Animals very well fed and plump: No immediate mating reaction and no conjugants present at the end of 24 hours.

(2) Animals well fed but not markedly plump: A weak immediate mating reaction; a few animals cling together in pairs but break apart in a short time; no conjugants present at the end of 24 hours.

(3) Animals of moderate size, not well fed: Strong mating reaction; many clumps form; these later disintegrate into pairs which remain together and complete conjugation.

(4) Animals small and thin: Strong mating reaction; many clumps form; these later disintegrate, but few or none of the animals proceed to conjugate.

(5) Animals very small and starved: No immediate or later mating reaction and no conjugation.

The mixtures used in the above observations were kept for only 24 hours so that it is not known whether conjugation would have occurred in the mixtures in the first two stages if they had been kept for a longer time, but it seems probable that conjugants would form as the paramecia reached stage 3. The various stages of nutritive decline can be seen successively in a culture to which a considerable amount of food is added and the culture then allowed to decline without the addition of more food.

These observations appear significant in that they indicate that the conditions under which the mating reaction occurs are not necessarily favorable for conjugation; i.e. the mating reaction is much less sensitive to nutritive conditions than conjugation so that the paramecia give the mating reaction before they have reached the proper condition for conjugation and also after they have passed this condition.

Diurnal Periodicity

Since a diurnal periodicity in the mating reaction has been found in certain groups of *P. bursaria* (Jennings, 1939*a*) and *P. aurelia* (Sonneborn, 1938*a*), and furthermore, since it has been stated by Maupas

(1889) that *P. caudatum* conjugates at about 4:00 A.M., this question was investigated with all five groups of mating types.

In the investigation of periodicity the mixtures were examined immediately for mating reactions and twelve hours later for conjugants. In Group 1 hourly mixtures with immediate mating reactions and later conjugation were made only between 11 P.M. and 6 A.M., but since pairs just beginning to form have been observed at various hours of the morning and afternoon, it seems fair to conclude that there is no diurnal periodicity in this group. In Groups 2 and 3, mixtures followed by immediate clumping and later conjugation were made at all hours of the day and night, so that it is obvious that there is no periodicity in these two groups. In Groups 4 and 5, immediate clumping and later pairing was obtained in mixtures made between 8 A.M. and 2 A.M. Although mixtures were not tried between 2 A.M. and 8 A.M., strong mating reactions were obtained at both ends of this period and it seems probable that clumping and conjugation would have occurred if mixture had been made at these times. It therefore appears that there is no diurnal periodicity in any of the five groups of mating types so far discovered in *P. caudatum.*

Temperature

It was desired to investigate the effect of temperature upon conjugation in order to ascertain what temperature was most favorable for conjugation, whether the temperature to which the paramecia had been exposed before mixture had any effect on the number of conjugants formed, and whether any differences existed among the groups in their response to temperature. In investigating this problem all factors except the temperatures used were kept as constant as possible. One representative clone of each mating type of Group 1, Group 2, and Group 3 was used in this work. Four cultures of each mating type were left at room temperature (24° to 28° C.) for two days. At the end of this time, additional culture fluid was added and they were placed at the various temperatures—in Groups 1 and 3: 9°, 18°, 24°, and 31° C., in Group 2: 9°, 20°, 24°, and 28° C. The cultures were left at the various temperatures for forty-eight hours. For Groups 1 and 3, at the end of this time four mixtures of the two types from each temperature were placed at all the temperatures used. Thus, four mixtures from 18° were placed at 9°, four at 18°, four at 24°, and four at 31° C., and in the same manner for each of the other temperatures. In the case of Group 3 the experiment was repeated, giving a total of eight mixtures in all. In no case were any mixtures made between cultures which had previously been kept at two different temperatures; all were between two cultures kept at the same temperature. In Group 2, six mixtures were made instead of four and the experiment was performed twice, giving twelve mixtures in all. The mixtures were examined at twelve-hour intervals and all conjugants present were removed with a pipette and the number present recorded. When no conjugants had been found in the mixtures for the three previous twelve-hour periods, the remaining animals were removed and counted. This number was used in calculating the percentage of conjugation.

The results of the experiments are given in Table II. In Group 2, it will be seen that the greatest percentage of conjugation occurred when animals kept at 20° C. were put after mixture at 20° C. In general, the results indicate that the temperature at which the animals were kept both before and after mixture affect the amount of conjugation. Either very high or very low temperatures after mixture either decrease the percentage of conjugation markedly or prevent it entirely. Low temperatures (9° or 20°) before mixture appear to be more favorable for conjugation than high temperatures (26° or 28°).

TABLE II

The effect of various temperatures on the number of pairs of conjugants and the percentage of conjugation in mixtures of Group 2 and Group 3 animals. The means of eight or twelve mixtures are given in the table.

		Group 2							
		Temperature after mixture of the two types							
		28°		26°		20°		9°	
Temperature for two days before mixture	28°	1.0	2.2%	8.6	18.3%	15.0	29.4%	0	0%
	26°	2.0	3.5%	8.3	18.3%	16.4	31.8%	0	0%
	20°	4.2	6.7%	19.4	33.0%	33.5	54.8%	0	0%
	9°	0.0	0.0%	19.7	27.5%	33.7	45.5%	0	0%
		Group 3							
		31°		24°		18°		9°	
	31°	4.5	9.0%	73.8	86.7%	63.2	88.2%	0	0%
	24°	0.0	0.0%	68.2	88.5%	54.5	83.8%	0	0%
	18°	0.0	0.0%	61.2	78.5%	49.5	71.2%	0	0%
	9°	0.0	0.0%	53.2	63.7%	42.2	49.7%	0	0%

In Group 3, the highest percentage of conjugation occurred when mixtures of animals from 24° C. were kept at 24° C. In general, it can be said that, as in Group 2, very high or low temperatures after mixture are unfavorable for conjugation. Unlike Group 2, high temperatures before mixture appear to be more favorable than low.

When the numbers of conjugants formed in each of the mixtures during each of the twelve-hour periods after mixture until no more conjugants were formed were considered, it was found that in Group 2 most of the conjugants were formed in the first twelve hours after mixture. In only one set of mixtures,—those put at 20° after two days unmixed at 9°,—is the time of greatest conjugation shifted to the period between twelve and twenty-four hours after mixture. At 24° in Group 3 most of the conjugants were found between twelve and twenty-four hours after mixture while at 18° most of the conjugants were found 36 to 48 hours after mixture.

In Group 1, the results obtained are not complete enough to justify inclusion but they indicate that the lower the temperature before mixture the greater the conjugation, and that most conjugants are produced when the paramecia previously kept at 9° C. are put at 20° C.

Group Differences

An attempt was made to ascertain whether there were any differences between the groups besides the primary group difference in the mating type. With respect to size, it was found that the animals in Group 3 are characteristically smaller than the animals in Groups 1, 2, 4 and 5. Under isolation line conditions, these differences are hardly noticeable but when grown in mass cultures become very striking. In Group 1, it was found that on the average the type II animals were smaller than the type I and of a slightly different shape, being rather shorter and broader. This difference becomes very obvious in conjugating pairs where the type I members may frequently be twice as long as the type II member. So far only two collections of eight clones in all have been found for Group 1 so that it is possible that the condition is not general for the group. However, all the clones so far examined (three of type II and five of type I) show the difference. In Group 2 characteristic size differences between clones have been observed but without any correlation with mating type.

There are also characteristic differences in Group 1 as to selfing. Type II animals self readily while the type I animals self very rarely. In Group 2, both selfing and non-selfing clones are found but they are not correlated with mating type. Group 3 seems to have rather less selfing than either of the other two groups since only a few instances of conjugation without mixture have been found in this group.

Under good conditions for conjugation, there are, as mentioned previously, differences in the proportions of the animals in mixtures which conjugate. Thus the greatest proportion of conjugation in mixtures occurred in Group 3 and the lowest proportion of conjugation in

Group 1. Another already-mentioned difference with regard to conjugation is that low temperature before mixture causes more conjugation in Groups 1 and 2 but less conjugation in Group 3.

A comparative study was made of the adverse effects on *P. caudatum* of mixture with three clones of *P. aurelia* (H, G, and 47). These clones are known (Sonneborn, 1938*b*, 1939, and unpublished) to cause certain other clones of *P. aurelia* to die in characteristic fashion when they are mixed with them. These three clones were mixed with 73 clones of *P. caudatum* and the mixtures were observed daily until dead or until all the other mixtures which showed an effect were dead. In all cases, control groups of *P. caudatum* were kept without mixture with the clones of *P. aurelia.*

The effect on *P. caudatum* which is produced by G is first indicated by the avoiding reaction and spinning by the affected individuals. The spinning takes the form of rapid rotation on the longitudinal axis with little or no forward movement. In some instances, the animals revolve (without moving forward) around an axis parallel to the longitudinal axis of the paramecium so as to describe a cylinder or a segment of a cone. These manifestations do not occur continuously but alternate with periods of quiescence or normal swimming. In addition to the alteration in behavior described above, morphological changes also occur; the paramecia become thin, flattened and gradually become transparent. As they become transparent, crystals become visible in the cytoplasm. There is no regularity about the position of the crystals, which are sometimes in the anterior end, sometimes in the posterior end, and sometimes distributed about the periphery. No characteristic differences in the reaction to G were noted between the groups of mating types.

Stock H has no marked effect on behavior of *P. caudatum* but the morphological changes are much more striking than those produced by Stock G. The affected animals stop feeding and lose all their food vacuoles. They then become filled with clear vacuoles. Generally there were two large vacuoles in each animal in the region of the contractile vacuoles and several smaller vacuoles. In some, only one large vacuole was formed. The vacuoles gradually enlarge until the ectoplasm becomes widely separated from the rest of the cytoplasm and the paramecia appear blistered. Frequently, just before death, the macronucleus becomes visible as a round body. In many dead animals both the blistering and the visible macronucleus are evident while in others only one is to be seen. In other cases, only the vacuolization was apparent. No characteristic differences were noted among the first three groups in their response. However, both Groups 4 and 5 were resistant to the effect of H and remained normal.

The effect of Stock 47 was as striking as that of H in a somewhat different way. The first effect was that the animals became shorter and thicker than normal. This was followed by enlargement of the posterior end. This enlargement appeared to be caused by the massing of the cytoplasm and macronucleus at the posterior end toward one side of the animal. The animals frequently became almost spherical before death. In some instances a single vacuole appeared at the posterior end but this was not a constant effect. Much more common were small vacuoles under the ectoplasm giving the animals a rough appearance. None of the clones tested were resistant to Stock 47, and it is of considerable interest that the clones which were resistant to H appeared to be more quickly and strikingly affected than the other clones.

In summary, it can be said that there appear to be characteristic differences between the groups in size and in various physiological characteristics. In Group 1, there also appear to be characteristic differences between the two mating types in size. In the other groups, there were no such characteristic differences between the mating types.

Discussion

The condition in *P. caudatum* with respect to the number of types within a group is like that in *P. aurelia,* since only two mating types have been found in each group; not four or eight as in *P. bursaria. P. caudatum* differs from both *P. aurelia* and *P. bursaria* in that it has certainly four and in all probability five groups of mating types while they have only three groups.

The wide occurrence, under certain conditions, of conjugation within a clone is of considerable interest in connection with the question of whether or not the presence of both mating types is necessary for conjugation to occur. In one clone (M) when pairs were split, it was found that both mating types of the group were indeed present. This may have been due to a change of mating type at autogamy, such as was found to occur in *P. aurelia* by Sonneborn (1937) and Kimball (1937). The possibility of accidental contamination cannot be excluded, however. In all the other clones of *P. caudatum* in which it was possible to separate conjugating pairs, the cultures derived from both members of a split pair were of the same type as the original culture. It has been impossible as yet to ascertain whether two mating types were present at the time conjugation occurred, one of which changed type again to become the same as the original clone or whether conjugation was occurring between two animals of the same mating type. Kimball (1939) has shown that in *P. aurelia* there may be a temporary change of mating type during vegetative reproduction but that animals which have thus changed their mating type give rise to clones of the original mating type. It appears possible that a similar process occurs in *P. caudatum*. That the repeated occurrence of conjugation within a clone was the result of a preceding autogamy appears unlikely in view of the fact that the two members of split pairs gave rise to clones of the same mating type and in view of the failure to find evidence of nuclear change in these clones. In some clones selfing occurred frequently while in others it occurred only once or not at all. This difference between clones is probably the basis of the conflicting results on the effect of environmental changes on conjugation in *P. caudatum*. Zweibaum (1912) found that he could induce conjugation in the clone of *P. caudatum* with which he was working by adding various salts in certain concentrations. He concluded, since he used only this one clone, that conjugation was dependent solely on environmental factors. Hopkins (1921) and Ball (1925) tried Zweibaum's methods on a number of clones of *P. caudatum* and found that they gave conjugation with some clones but not with others. These conflicting results can be explained by assuming that Zweibaum had a clone of animals in which selfing occurred rather readily, while Hopkins and Ball, since they used several clones, had some which selfed readily and some which would not self.

The nutritive requirements observed for conjugation appear to agree very well with those reported by Maupas (1889), Calkins and Cull (1907), Jennings (1910), Zweibaum (1912), Calkins and Gregory (1913), Ball (1925), Chatton and Chatton (1931), and Giese (1935). The paramecia conjugate when not too well fed, yet not starved. This is, of course, the condition produced when the food supply is suddenly decreased or when animals which have exhausted the food in the medium are given a limited supply of food. The most interesting result of the work on the effect of nutrition on conjugation is the fact that the mating reaction, clinging together and clumping, occur under conditions which are not satisfactory for conjugation. This phenomenon of strong clumping with the production of few or no pairs of conjugants was observed in several mixtures of clones in Groups 1 and 2. It is especially marked in those animals which have passed the point of optimum nutritional condition for conjugation and somewhat less so when the animals have not yet reached this condition.

SUMMARY

1. An investigation of the numbers and interrelations of the mating types in *Paramecium caudatum* in cultures derived from single animals

isolated from wild cultures was carried out. Of 93 clones from 26 natural sources the mating types are still to be identified in 3 clones from 2 natural sources.

2. The clone cultures could be divided into mating types in several non-interbreeding groups. Animals from cultures of different groups did not conjugate when mixed with one another. Within each group two mating types were found. Animals from cultures of different mating types belonging to the same group conjugated when mixed together. Four non-interbreeding groups of mating types have been definitely established and the occurrence of five groups is highly probable.

3. In regard to the geographical distribution of the mating types, no evidence was found for the formation of local groups of mating types which would not conjugate with animals from other localities.

4. Ordinarily, conjugation occurred only when animals from two different mating types were mixed but under certain conditions some clones conjugated without mixture.

5. It was found that ordinarily such conjugation was not the result of the production of two mating types at autogamy as in *P. aurelia.*

6. In one case (clone M, type III) both mating types of a group were produced in a clone but it was not possible to correlate this fact with a preceding autogamy.

7. It was found that the mating reaction itself (clumping of the animals) would occur under nutritive conditions which would not permit the completion of conjugation.

8. The temperature both before and after mixing the mating types has a definite effect on the proportions of the animals conjugating. Very little or no conjugation occurred when the animals were kept at the extremes of temperature (9° and 28° and 31°) after mixture. A low temperature prior to mixture caused more conjugation in Group 2, less in Group 3.

9. None of the five groups of mating types gave any indications of a diurnal periodicity.

10. Group 3 animals are obviously smaller than Group 1 or Group 2 animals.

11. In Group 1, there is a difference in size between the mating types; type I is larger than type II.

12. Type II animals "self" (conjugate without mixture) much more frequently than type I animals.

13. In mixtures between animals of different types the largest percentage conjugate in Group 3 and the smallest in Group 1.

14. No differences were found between Group 1, 2, and 3 in their response to the toxic effects produced by races G, H, and 47 of *P.*

aurelia. The clones which form Groups 4 and 5 are resistant to the lethal effect of H.

15. A possible explanation of the conflicting results on the effect of environmental factors on conjugation obtained by earlier workers on *P. caudatum* was presented.

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THE FUNCTION OF THE ANTENNAL RECEPTORS IN LEPIDOPTEROUS LARVAE[1]

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The problem of correlating the different structural types of sensilla with the various sensory perceptions recognized in insects has, with the exception of vision and the mechanical senses, met with only fair success. Olfactory sensilla are still so called primarily because of their structure. Two of the greatest difficulties encountered in determining experimentally the function of certain sensilla have been their wide distribution over the body and the proximity of many different types on a single appendage or given area. In this respect the antennae of lepidopterous larvae are ideal organs for study since the sensilla thereon are ten in number and comprise only five structural types. Advantage has been taken of this situation to study not only the functions of the antenna but also to attempt to assign some function to each of the various sensilla located there.

Two sensory faculties have been ascribed to the larval antenna. Blanc (1889) considered it simply a tactile organ. Nägel (1897) maintained that it was primarily an olfactory organ. Both of these authors based their assumptions on structure alone. The purpose of this paper is to point out the probable functions of the antenna as indicated by experiments on several species of caterpillars and to attempt to correlate this sensory activity with the five morphological types of sensilla borne there.

Material and Methods

The species of caterpillars employed were determined largely by their seasonal and numerical availability. Since the antenna is fundamentally the same in all larvae of this order (cf. Dethier, 1941) and the reactions predictable irrespective of the species used, it was deemed unnecessary to restrict experiments to a single species. Similarly interspecific comparisons seemed allowable. Third, fourth, and fifth instar larvae of the following species were employed: *Isia isabella* A. & S. (Arctiidae), *Liparis dispar* L. (Liparidae), *Malacosoma disstria* Hbn. (Lasiocampidae), *Cingilia catenaria* Dru. (Geometridae), *Pieris rapae* L. (Pieridae), and *Nymphalis antiopa* L. (Nymphalidae). It soon became apparent that age had no effect on the results obtained, hence larvae that had attained size convenient for dissection became fit subjects for experimentation. Approximately five hundred specimens were used.

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[1] The writer wishes to express his appreciation to Professor C. Ladd Prosser of the Department of Biology, University of Illinois, for his generous assistance in the electrical phases of this problem.

It was proposed: (1) to record and measure action potentials on the antennal nerve when the long hairs were stimulated by bending; (2) to study the character of the olfactory sense and ascertain the effect of antennal extirpation on the general threshold for response to odors; and (3) to record and measure action potentials on the antennal nerve when the end organs were stimulated by odorous substances.

Action potentials on the antennal nerve were recorded photographically by means of a Matthews oscillograph and a resistance-capacity coupled amplifier. Conduction of the experiment was rendered difficult by reason of the toughness of the head capsule and general inaccessibility of the antennal nerve as well as the extreme shortness of the nerve. A larva to be tested was fastened ventral side down to a block of paraffin by means of fine insect pins. The anterior surface of the head capsule comprising the vertex and adfrontal areas was excised with a pair of iridectomy scissors. Removal of this piece of cuticle left a window in the front of the head capsule exposing the brain and its nerves. Experience showed that neither Ringer's solution nor a moist chamber preparation were necessary because there was practically no drying out of the nerves during the first hour of experimentation. After one hour had elapsed, a fresh specimen was prepared. Due to the shortness of the nerve (1.75 mm.), all recordings were mono-polar. A fine silver wire hooked under the nerve raised it into the air. A thicker silver wire led down into the tissues and body juices of the head. This lead was used alternately as ground and grid. Unfortunately, recordings of this nature rendered impossible any interpretation of wave form, and spike heights were purely relative.

The general threshold for response to olfactory stimuli was measured with a specially designed olfactometer. Caterpillars give no recognizable response to attractant odors nor do they respond to repellent odors unless the concentration is high. For these reasons it was necessary to stimulate with repellent odors and to deliver measured concentrations directly to the larvae.

A stream of nitrogen gas of constant velocity was saturated with the test odor by being bubbled through a C. P. grade of the odorous liquid. It was then diluted to the desired concentrations by the addition of oxygen gas and the resultant mixture delivered to the chamber containing the larva. Complete saturation of the nitrogen was insured by the use of three all-glass saturators in series. These were adaptations of the type designed by v. Bichowsky and Storch (1915). The flow of gas in each case was regulated by needle valves and measured by glass flow meters (Benton, 1919). The gases were mixed in a chamber which also acted as a valve preventing the more rapidly flowing gas from blocking the flow of the less rapidly flowing one. A larva was confined in a glass tube 8 mm. in diameter and 45 mm. long. A cap of fine-mesh copper screening at either end prevented the escape of the animal yet allowed the free passage through the tube of all gases. This tube was then placed within the glass test chamber to which the mixed gases were delivered and from which they escaped into the room. Saturators, mixing chamber, and test chamber were immersed in a constant temperature bath maintained at 20° C. This temperature was found to be optimum for larvae and convenient for calculations of concentrations. A light was suspended over the test chamber to illuminate the animal and cause it to remain in a central position where it was observed with the aid of a large magnifying glass clamped over the chamber. All connections were glass to glass. Rubber tubing and stoppers, even when treated, emitted odors. Cork stoppers were used wherever frequent breaking of connections was necessary. They were replaced several times daily.

Inability to compress or secure compressed air necessitated the use of a cheap, easily obtained gas. Nitrogen filled these requirements. The addition of oxygen was necessary for the maintenance of life. Two variables, the oxygen/nitrogen ratio and the gas velocity, constituted possible sources of error. Carefully controlled experiments indicated that changes in the oxygen concentration of the gas mixture ranging from 20 per cent to 100 per cent oxygen had no effect on the response to odor. The average thresholds for larvae maintained at atmospheric concentrations of oxygen did not differ significantly from those for larvae maintained in a gas mixture containing about 99 per cent oxygen. Therefore, the small changes in oxygen concentration introduced in the course of the experiments (80 per cent to 99 per cent) required no further control. Responses to variations in gas velocity accompanied large and abrupt changes only. Thresholds could be determined at different gas mixture velocities since concentrations depended upon the ratio of the rate of flow of oxygen to the rate of flow of nitrogen and not on the total velocity. Thus it was found that the threshold of response to odors remained unaffected by small velocity changes occurring under usual experimental conditions. Concentrations were calculated in terms of grams of solution (in this case benzaldehyde) per liter of gas mixture from the following equation:

$$W = \frac{p_a \cdot M_a}{\left[(P_b - p_a)RT\right]\left[F_{O_2} - \left(\frac{P_b}{P_b - p_a}\right)F_{N_2}\right]}$$

where W = the number of grams of benzaldehyde per liter of gas mixture, p_a = the vapor pressure of benzaldehyde in millimeters of mercury at the temperature of the solution, M_a = the molecular weight of the benzaldehyde, P_b = the barometric reading in millimeters of mercury, R = the gas constant (0.08207 liter atmospheres), T = the absolute temperature, F_{O_2} = the ratio of the rate of flow of oxygen to the rate of flow of nitrogen in liters per minute, and F_{N_2} = the rate of flow of nitrogen in liters per minute. Humidity was not controlled and may have had some slight effect on the threshold values.

The Antennae

The antennae are located on the ventral lateral surface of the head arising from the region of the postgenae near the bases of the mandibles.[2] They are inserted into a membranous area in the head capsule known as the antacoria. Each antenna is three-segmented (Fig. 1). The first or basal segment sometimes contains four sensilla campaniformia. Upon the second segment are located most of the antennal sensilla. At the proximal end approximately in line with the larger hair is located a single sensillum campaniformium. Next in order are two long thick-walled hairs (sensilla trichodea). They are true hairs arising from articulation sockets. Distally there are always three sensilla basiconica, two large and one minute. Segment three usually contains four sensilla apically, a sensillum styloconicum, a large sensillum basiconicum, and two small sensilla basiconica. The three large, hollow, thin-walled sensilla basiconica possess elaborately sculptured surfaces.

Three discrete sets of muscles inserted on the anterior mesal edge of the base of the proximal segment effect the withdrawal of the antenna. These muscles originate in the head capsule in the parietal region laterad of the adfrontal area. Extension of the antenna is regulated by blood pressure.

A single nerve from the deutocerebrum innervates the antenna. A basal branch terminates in the head capsule adjacent to the insertion of the antenna; the antennal branch innervates the antenna proper. Each of the two hairs is innervated by a single bipolar sense cell. Also innervated by single bipolar sense cells are the sensillum campaniformium and the small sensilla basiconica. Four bundles of primary bipolar sense cells fill the greater part of the antenna distally. Each of the three large sensilla basiconica is innervated by one bundle (Fig. 2). The fourth

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[2] A complete description of the antennae has been given in a previous communication (Dethier, 1941). Figures 1 and 2 are reproduced through the courtesy of the *Bulletin of the Museum of Comparative Zoölogy*, Harvard College.

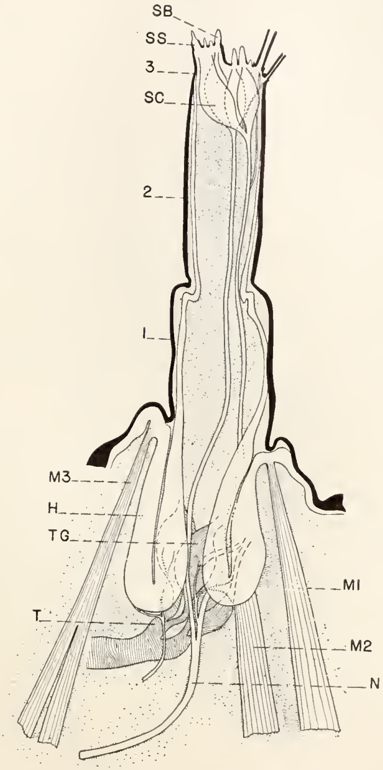

Fig. 1. Semidiagrammatic longitudinal section of the antenna (after Dethier, 1941). 1, 2 and 3, first, second and third segment respectively; *M*1, *M*2 and *M*3, first, second and third muscle bundle respectively; *T*, trachea; *N*, nerve; *SB*, sensillum basiconicum; *SS*, sensillum styloconicum; *SC*, primary bipolar sense cells. (This figure is reproduced by courtesy of the *Bulletin of the Museum of Comparative Zoology.*)

bundle apparently innervates sensilla on the third segment. As many as twenty-five cells have been counted in each bundle.

The Tactile Sense

Scattered over the bodies of caterpillars are numerous thick-walled hairs (sensilla trichodea) differing markedly in length and diameter. It

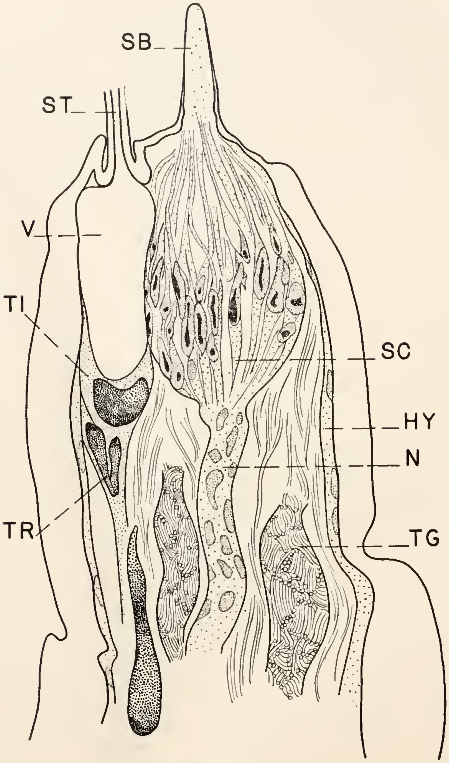

FIG. 2. Longitudinal section through the second antennal segment showing the innervation of the hair and sensillum basiconicum (after Dethier, 1941). *SB*, sensillum basiconicum; *ST*, sensillum trichodea; *TI*, trichogen; *TR*, tormogen; *V*, vacuole; *SC*, primary bipolar sense cells; *N*, nerve; *HY*, hypodermis; *TG*, tracheolar glomerulus. (This figure is reproduced by courtesy of the *Bulletin of the Museum of Comparative Zoology*.)

has been demonstrated repeatedly that many of these subserve a tactile function. The resemblance in structure of the two hairs characteristically found on the antenna to tactile hairs of the body very early led to the belief that they also were tactile hairs. The function of the remaining sensilla was largely ignored.

By observing directly the response to tactile stimulation of the different sensilla and by recording action potentials on the antennal nerve, it has been possible not only to verify the function of the large hairs but also to determine the functional nature of some of the other sensilla. Action currents were recorded by means of the apparatus and preparations already described. Hairs were stimulated first by touching gently with another hair from the body of the animal, second by bending with a fine glass needle. In the former case no responses were observed on the intact animal nor any action potentials recorded on the antennal nerve of the dissected animal. When the long hair was bent sufficiently to cause movement of the hair within its socket, the intact animal responded by quickly withdrawing the antenna into the antennal socket. Impulses were recorded when stimulation of this sort was applied to the dissected animal. Since the nerve was severed centrally, there were no spontaneous discharges nor motor activity from the brain. Spike heights averaged 8 microvolts. After the long hair was cut away at its base, stimulation of the short hair was possible. The tactile threshold of this hair, judging from the amount of bending necessary to produce a response, is much lower than that of the long hair. Following removal of the short hair the remaining sensilla were stimulated tactually without interference. Attempts were made to touch each sensillum individually. This proved exceptionally difficult. A micro-manipulator and fine glass needles were tried without success. Finally stimulation was accomplished by means of a fine hair from the animal's body. When the sensilla on the third antennal segment were touched, immediate withdrawal of the antenna resulted. It was impossible, however, to touch the large sensillum basiconicum without also touching the sensillum styloconicum. Attempts to touch either of the large sensilla basiconica on segment two without also stimulating the minute sensillum basiconicum were not entirely successful. It was possible with an animal possessing an abnormal antenna which lacked large sensilla basiconica to stimulate the minute sensilla basiconica. This much is certain. Light tactile stimulation of the minute sensilla basiconica resulted in a response; bending of the large sensilla basiconica sufficiently to cause strains in the surrounding surface cuticle also caused a response. No other part of the antenna appeared sensitive to tactile stimulation.

When the cuticle in the region of the sensillum campaniformium was deeply depressed by pressure with a glass needle, withdrawal of the antenna resulted. This region, however, was not sensitive to light touches. Movement of the antenna as a whole on the dissected animal with the nerve cut centrally resulted in a burst of impulses. These probably resulted from stimulation of the sensillum campaniformium since no muscle receptors have been found associated with the antennal muscles.

THE OLFACTORY SENSE

The existence of an olfactory sense in caterpillars was first demonstrated by McIndoo (1919). Larvae of several species responded to the odors of essential oils. End organs on the distal segments of the antennae and maxillae were suggested in a previous communication (Dethier, 1937) as the probable olfactory receptors. The ability of the larvae to respond to essential oils is impaired or destroyed by removal of these areas. Götz (1936) maintained that the olfactory sense was not localized in the antennae or maxillae insomuch as larvae continued to feed following extirpation of these appendages. This conclusion does not seem justifiable because the initiation of feeding is not always dependent upon chemotaxis. Caterpillars frequently attempt to eat odorless cellulose materials. They are known to eat their way to freedom when confined in cardboard containers.

TABLE I

Olfactory response and longevity of *Cingilia catenaria* following operations to the antennae and maxillae

Specimen no.	Date of operation	Response	Date of operation	Response	Date of pupation	Date of emergence
182	Maxillae removed 6/30/37	Excellent	Antennae removed 7/3/37	None	7/25/37	8/8/37
197	Antennae removed 6/30/37	Excellent	Maxillae removed 7/9/37	None	7/15/37	7/28/37
198	Antennae and maxillae removed 7/1/37	None	—	—	7/12/37	7/26/37

Repeated experiments have shown that extirpation of the antennae does not abolish responses to essential oils or odorous liquids held close to the head. Nor does removal of the maxillae completely destroy the olfactory sense. Only when both pairs of appendages are removed is the sense of smell destroyed. As long as the operation was carefully executed a larva suffered no permanent ill effects. Parts were removed by cutting with a microscalpel. Recovery from surgical shock was complete thirty minutes after cutting. As a precautionary measure, however, experiments were not conducted until twenty-four hours had elapsed. Best results were obtained without the use of anesthetics. Two sets of experiments demonstrated that failure to respond to odors following removal of both antennae and maxillae was not due to more severe surgical shock than the removal of either pair alone. (1) Regeneration of antennae or maxillae in subsequent instars was accompanied by return of the olfactory sense. (2) Operations involving injury to more and larger nerves, as removal of all thoracic legs or decapitation, did not result in shock of sufficient severity to impair the olfactory sense. Mortality rates were less than 1 per cent. It may be seen from Table I that these operations were not sufficiently shocking to interfere with the normal life processes of the insect.

Two facts were noted in the course of these experiments which suggested that a determination of the threshold for response to olfactory stimuli might shed further light on the nature and locus of the olfactory sense. (1) Larvae lacking maxillae apparently responded more rapidly than larvae lacking antennae. (2) Occasional specimens lacking both antennae and maxillae gave questionable, feeble responses when strong odorous liquids, as turpentine or ammonia, were held not more than one millimeter away from the head for a period of sixty seconds or longer.

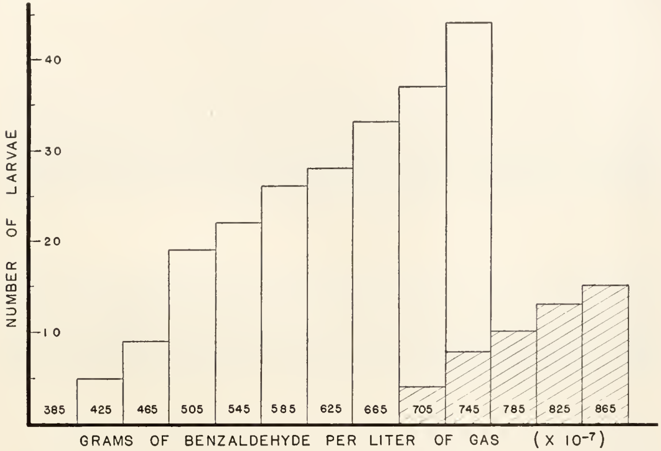

FIG. 3. Change in the threshold sensitivity of larvae of *P. rapae* L. to the odor of benzaldehyde following amputation of the antennae. The open histogram represents the threshold of response of normal larvae; the cross-hatched histogram, that of larvae lacking antennae.

Accordingly the threshold values for response to benzaldehyde were determined for normal larvae of *Pieris rapae* as described above. It was found that the average threshold for response was $580 \times 10^{-7} \pm 16.49 \times 10^{-7}$ grams of benzaldehyde per liter of gas mixture. For larvae from which the antennae had been removed the threshold was

$770 \times 10^{-7} \pm 3.7 \times 10^{-7}$ grams per liter. This difference in the average thresholds is eleven times the standard error of the difference (Fig. 3). No significant rise in threshold could be demonstrated for larvae from which the maxillae had been removed. Unilateral extirpation of either appendage likewise caused no rise in the threshold value. This would seem to indicate, first that the antennae did actually possess olfactory end organs, and second that the olfactory threshold of the antennal end organs was lower than that of the maxillary end organs.

In 0.5 per cent of all of the tests on animals lacking both antennae and maxillae a response to benzaldehyde could be obtained. The lower limit of the threshold for response in these cases was 1×10^{-3} grams per liter or approximately 17 times the same value for a normal individual. Although this could be interpreted as meaning that olfactory end organs are also borne on parts of the body other than the antennae and maxillae, it seems more probable that odors in such high concentrations acted as irritants and were stimulating organs similar in nature to the common chemical sense of man.

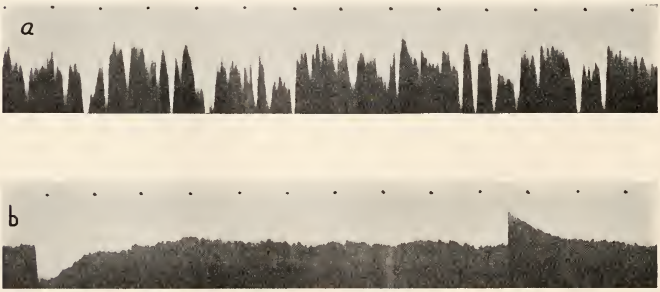

FIG. 4. *a.* Record of responses on antennal nerve to stimulation of tactile hairs on antenna. Time signal at top of record shows intervals of 0.01 second.

b. Amplifier baseline with calibrating signal of 8 microvolts. Time signal, 0.01 second.

A further effort was made to confirm these findings by recording action potentials on the antennal nerve when the receptors on the tip of the antenna were stimulated by strong odors applied from a short distance. Turpentine, oil of wintergreen, oil of cloves, and benzaldehyde, which cause the animal to react under normal conditions, were used as test substances. Recording methods and preparations were as described above.

With this preparation no action potentials were recorded upon stimulation of the antennal sensilla. Spikes due to accidental tactile stimulation of the hairs on the tip of the antenna did appear on the record. They were of the order of magnitude of 8 microvolts. Failure to detect potentials from chemoreceptors was thought to be due to the fact that the potentials from these receptors were of too low a magnitude to be distinguished from the ordinary fluctuations of the base line. That this was actually the case is proven below by application of Erlanger and Gasser's equation which states that the recorded potential of action varies as the square of the outside diameter of the axon.

A study of longitudinal as well as cross-sections of the antenna has shown that the largest sensory nerve fibers in the antenna innervate the tactile hairs. The average outside diameter of these fibers is 3 micra. The average diameter of the remaining fibers is 0.8 micra. The amplitude of spikes resulting from stimulation of the tactile hairs was of the order of magnitude of 8 microvolts. Measurements of spike amplitude were taken on a calibrated record of potentials from this nerve. Application of these values to the equation shows that the value of spikes from any of the other fibers would be of the order of 0.57 microvolts.

$$\frac{D_1^2}{P_1} = \frac{D_2^2}{P_2} \qquad \frac{0.64}{x} = \frac{9}{8}$$

With the electrodes far apart on fine nerves raised into the air resistance was exceedingly high. As a result there was a high noise level of the order of magnitude of 2 microvolts. Thus it was impossible to distinguish potentials of the order of magnitude of 0.5 or even 1.0 microvolt when the fluctuations of the base line approximated 2.0 microvolts.

Discussion

The present experiments confirm the assumptions of earlier workers that the antennae of lepidopterous larvae are tactile and olfactory organs. All of the evidence favoring the tactile nature of the large sensilla trichodea is direct. It is hardly necessary to add that these hairs by their structure are ideally adapted for the reception of tactile stimuli. Any object or vibration impinging upon either hair of sufficient magnitude to cause movement of the shaft within its socket may be an adequate stimulus. Of such a nature are air currents, vibrations of the substratum, and preëminently, shocks imparted to the hairs by exploratory movements of the antenna. Stimuli of this last sort serve to inform the animal of obstacles in its path and of the contours of the substratum. In this respect other hairs on the feet and mouthparts are of nearly equal importance. Finally the antennal hairs protect the more delicate antennal sensilla in that by stimulation thereof the animal is made aware of potentially injurious objects. The antennal hairs are not alone in this service. Stimulation of numerous structurally similar tactile hairs on the parietal region of the head capsule results in withdrawal of the antenna.

It is to be expected that the longer hair would have the higher threshold since it is continuously stimulated. Stimulation of the short hair signals proximate danger to the antenna.

Direct evidence likewise indicates that the sensillum campaniformium responds to bending of the adjacent cuticle. It is likely that this sensillum is a proprioceptive organ. Movement of the antenna within its socket results in a burst of impulses on the antennal nerve (severed centrally). These undoubtedly originate with the sensillum campaniformium or with the minute nerves ending freely in the region of the hypodermal bulb. These may also be proprioceptive in nature.

The olfactory nature of the large sensilla basiconica still rests on indirect evidence. All experiments point to the olfactory function of the antenna. By a process of elimination the large sensilla basiconica must be olfactory end organs. Responses to tactile stimuli are observed, however, when these end organs are bent with a hair. Some doubt remains as to whether the response is due to direct stimulation of the sensilla basiconica or to transmission of the stimulus through the surrounding cuticle to the small tactile sensilla basiconica. It is also possible that in the aggregate of sense cells innervating the large sensilla basiconica there are tactile receptors as well as chemoreceptors. In other words, a large sensillum basiconicum may be a composite end organ. Histological examination, however, reveals no differences among the cells.

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STUDIES ON THE LIFE HISTORY OF ANISOPORUS MANTERI HUNNINEN AND CABLE, 1940 (TREMATODA: ALLOCREADIIDAE)

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INTRODUCTION

In August, 1939, the authors discovered a new cotylomicrocercous cercaria emerging from the marine snail, *Mitrella lunata* (Say) collected at Waquoit Bay, Cape Cod, Massachusetts. During the summer of 1940, it was found that this cercaria penetrated marine amphipods and developed into progenetic metacercariae of a new species of *Anisoporus,* for which the name *Anisoporus manteri* was proposed in a preliminary abstract (Hunninen and Cable, 1940).

The genus *Anisoporus* was erected by Ozaki (1928) to contain *A. cobraeformis* from the intestine of *Diacocus petersoni.* Two other species of *Anisoporus* from marine fishes have been described by Manter (1940),—*A. eucinostomi* and *A. thyrinopsi* which is described tentatively, being possibly a developmental stage of *A. eucinostomi. Anisoporus* possesses a small accessory sucker anterior to the acetabulum, thereby differing from the closely related genera *Opecoelus* and *Opegaster.*

There has been considerable difference of opinion concerning the taxonomic significance of anal openings such as those found in *Anisoporus, Opecoelus, Opegaster* and several other genera of digenetic trematodes. Ozaki (1925) regarded anal openings as fundamental characters and accordingly proposed the family Opecoelidae to include the genera *Opecoelus* and *Coitocaecum.* Later (1928), he placed the new genera *Anisoporus* and *Opegaster* in the Opecoelidae but removed *Coitocaecum,* making it the type of a new family, Coitocaecidae. In the same paper, he also erected the family Diploproctodaeidae, with *Diploproctodaeum* La Rue as type genus and included two new species of the new genus *Diploporus.* Odhner (1928) erected the genus *Opecoeloides* and expressed the opinion that *Opecoelus* and *Opecoeloides,* because of their resemblance to *Podocotyle,* were aberrant allocreadiids. Ozaki (1929) reaffirmed his belief that anal openings were fundamental

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characters. Stunkard (1931) has reviewed the literature concerning trematodes with anal openings and expressed the opinion that these structures have arisen independently in several families and are not of great taxonomic importance. He stated that the members of Ozaki's families Opecoelidae and Coitocaecidae might well be regarded as a subfamily, Opecoelinae, of the family Allocreadiidae. La Rue (1938) concludes that anal openings have little more than specific or generic value at the most in the taxonomy of digenetic trematodes.

The described species of freshwater and marine cotylomicrocercous cercariae and the life histories of trematodes known to have this type of larva are listed by Cable (1938, 1939) and Dobrovolny (1939*a*).

Materials and Methods

Material was collected in abundance from Waquoit Bay, near Woods Hole, Massachusetts, and studied mostly while living. Stained whole mounts and serial sections of certain stages were prepared, using conventional technics. Cercariae were studied with the aid of neutral red and Nile blue sulphate supravital stains. The morphology of the metacercaria and adult was observed in specimens from both naturally and experimentally infected amphipods and fishes. Amphipods were infected experimentally by placing them with infected snails in finger bowls which were covered with cheese-cloth and placed in slowly running sea water. All measurements given below are in millimeters. Eleven adults were measured as stained whole mounts; all other measurements were made on living material under light cover-glass pressure.

Observations

Experimental Proof of the Life History

Technical difficulties and brevity of the season made it impractical to rear parasite-free amphipods and fishes in the laboratory. For this reason, proof of the life history is based on the fact that penetration of the cercariae into amphipods could be induced at will and subsequent development followed as a continuous process from very young to mature, progenetic metacercariae with eggs in the uterus and discharged from the body into the surrounding cystic fluid. Amphipods exposed to cercariae in the laboratory usually contained a larger number of metacercariae and always showed a much higher incidence of infection than did amphipods collected in the field. Experimental infections superimposed on natural infections could be detected by differences in cyst size.

Fishes were infected experimentally by feeding them large numbers of amphipods. Worms recovered from experimentally as well as naturally infected fishes were little further developed than were the progenetic metacercariae.

Description of Stages in the Life Cycle

Adult (Figs. 7–10)

Specific Diagnosis.—Small, elongate worms with characters of the genus *Anisoporus*. Total length 0.74–2.32 (average 1.54); width 0.28–.46 (0.35). Oral sucker width 0.07–.14 (0.114); acetabulum in anterior third of body, 0.12–.17 (0.144) wide, provided with three anterior and two posterior papillae; sucker ratio approximately 2 : 2.5. Prepharynx very short, pharynx spherical, 0.07–.11 (0.09) in diameter; esophagus length 0.07–.18 (0.124); ceca extend almost to posterior end of body, uniting with excretory bladder, the excretory pore functioning as an anal opening. Testes tandem, in posterior half of body; anterior testis 0.13–.2 (0.16) wide and 0.07–.16 (0.12) long; posterior testis 0.14–.2 (0.18) by 0.07–.17 (0.13); cirrus sac lacking. Ovary ovoid, median, anterior to testes, 0.09–.14 (0.11) wide and 0.05–.12 (0.09) long; seminal receptacle lacking; Laurer's canal present. Uterus anterior to ovary, with few coils; vitelline follicles large, beginning just behind acetabulum and extending to posterior end of body, the lateral fields coalescing posteriorly; two accessory vitelline ducts extending transversely, one anterior to ovary, the other posterior to testes. Eggs 0.062–.068 (0.065) by 0.035–.04 (0.038). Excretory vesicle sac-shaped; excretory formula $2[(2+2)+(2+2)]$.

Hosts.—Northern pipefish, *Syngnathus fuscus* Storer; flounder, *Paralichthys dentatus* (Linnaeus); sand dab, *Hippoglossoides platessoides* (Fabricius); four-spined stickleback, *Apeltes quadracus* (Mitchill) and the killifishes, *Fundulus heteroclitus* (Linnaeus) and *F. majalis* (Walbaum).

Locality.—Waquoit Bay, Cape Cod, Massachusetts, U. S. A.

Type Specimens.—Holotype No. 36781 and Paratype 36782, Helminthological Collection, U. S. National Museum.

The body is elongate, tapering slightly at both ends. The cuticula is aspinose and is modified near the anterior end of the body to form small papillae (Fig. 9), each set with a very delicate "hair." The papillae are visible only in living material.

The ventral sucker is embedded in a large, stalk-like protrusion of the body, making it very difficult to mount worms so that the sucker is not displaced to one side. This displacement always causes distortion which alters the relationships of various structures. The characteristic lobes on the margin of the ventral sucker are shown in Fig. 6. The accessory sucker (Fig. 7) is seen more distinctly in living than in fixed and stained specimens and hence may have been overlooked in species at present assigned to genera other than *Anisoporus.* It is ventral in position, about midway between the acetabular stalk and the pharyngeal level, and appears to lie slightly to the left of the midventral line. The sucker has no connection with the genital pore which lies at the posterior end of the pharynx.

The shape of the oral sucker and pharynx depends on their state of contraction, being either subspherical or slightly wider than long. The short prepharynx is evident only in extended specimens. The esophagus bifurcates at the level of the acetabulum. In the living worm, a patch of tiny papillae is seen where the ceca join the bladder. These papillae are in the bladder proper and in sections resemble the inner processes of the muscle cells in *Ascaris.* They are especially noticeable during rhythmic contractions of the posterior end of the body.

The testes are intercecal; in moderately contracted worms, they lie close together, one behind the other, are definitely wider than long, and without notches or lobes. In extended specimens, the testes lie some distance apart and are spherical in shape. The vasa efferentia extend anteriorly and unite to form a very short vas deferens. The seminal vesicle is long, beginning well behind the acetabular level, almost as far back as the ovary in contracted specimens. The vesicle is continuous with a narrow, delicate ejaculatory duct which is difficult to trace as it approaches the genital pore.

The ovary lies in front of and in contact with the anterior testis. From the anterior surface of the ovary, the ciliated oviduct (Fig. 10) bends abruptly to the right, extends a short distance, then turns anteriorly and is joined immediately by the Laurer's canal. The canal crosses to the left of the median line and opens dorsally. From the junction of the oviduct and Laurer's canal, the oötype and uterus extend

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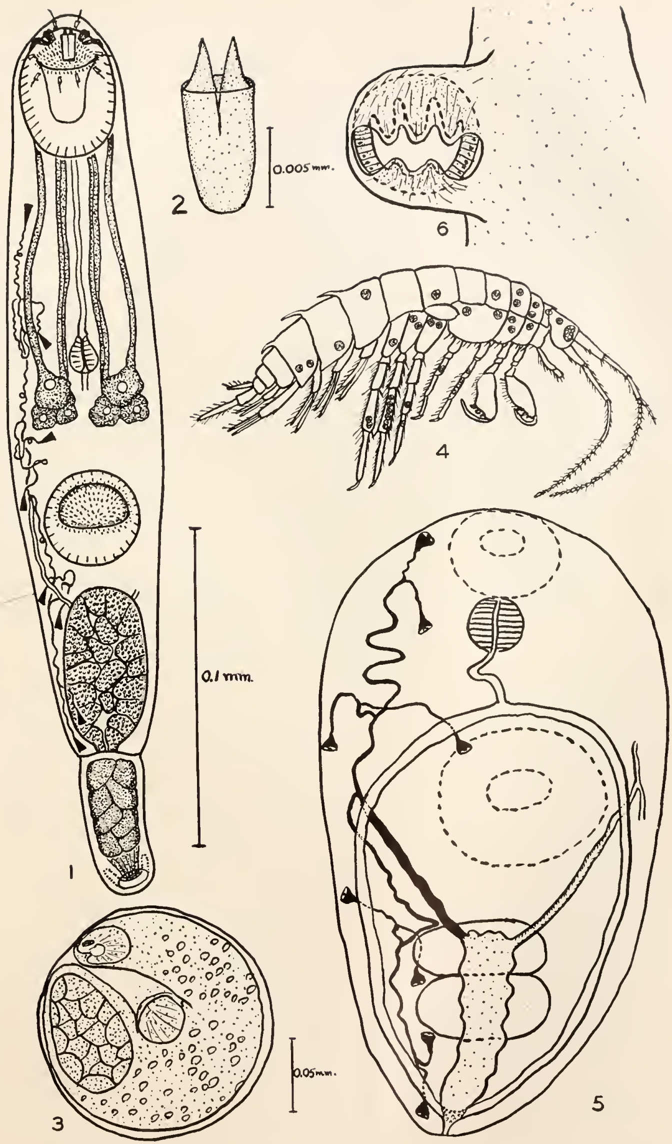
EXPLANATION OF PLATE I

(All figures concern *Anisoporus manteri*)

FIG. 1. Cercaria, ventral view.
FIG. 2. Stylet of cercaria, dorsal view.
FIG. 3. Metacercaria, 2-day infection.
FIG. 4. Amphipod with a moderately heavy infection with metacercariae.
FIG. 5. Metacercaria, showing excretory system and other details of structure.
FIG. 6. Ventral view of acetabulum, showing characteristic papillae.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3
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4
5
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PLATE I

anteriorly as a moderately sinuous tube. The uterus usually contains only a few eggs but as many as 80 have been counted. On each side of the body, an anterior and posterior vitelline duct join to form the transverse common vitelline duct which is expanded medially to form the vitelline reservoir. This reservoir joins the oötype just posterior to Mehlis' gland. The longitudinal vitelline ducts are connected by a pair of transverse accessory ducts (Fig. 7), one anterior to the ovary, the other posterior to the testes. These transverse ducts are clearly visible only when filled with vitelline material and may be overlooked when empty.

The excretory vesicle is a long, simple tube with small cells scattered over its inner surface. It extends to the anterior border of the anterior testis. The main excretory tubules are ciliated for about three-fourths their length and reach from the anterior end of the vesicle almost to the acetabular level where each divides to form an anterior and a posterior collecting tubule. Each collecting tubule receives two secondary tubules, each of which is joined by the capillaries of two flame cells. The excretory formula remains unchanged during post-cercarial development. The flame cells are large, averaging 0.012 mm. in length.

Anisoporus manteri is compared with described species of *Anisoporus* in Table I. *A. manteri* differs significantly from *A. cobraeformis* in

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Explanation of Plate II

(All figures concern *Anisoporus manteri*)

Fig. 7. Ventral view of an extended adult specimen (vitelline follicles omitted).

Fig. 8. Sagittal section of adult, showing junction of intestinal ceca and excretory vesicle.

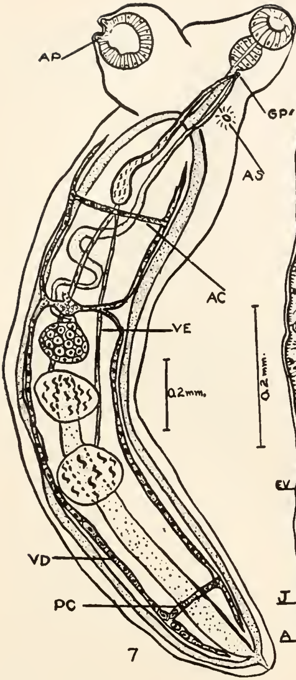
Fig. 9. Dorsal view of adult specimen.

Fig. 10. Details of reproductive system, drawn freehand from living specimen.

Fig. 11. Amphipod appendage containing five metacercariae.

Abbreviations

A, anus.
AC, anterior vitelline commissure.
AP, acetabular papillae.
AS, accessory sucker.
E, esophagus.
EG, egg.
EV, excretory vesicle.
GP, genital pore.
J, junction of ceca and excretory vesicle.
LC, Laurer's canal.
MG, Mehlis' gland.
OO, ootype.
OV, ovary.
PC, posterior vitelline commissure.
PH, pharynx.
PP, prepharynx.
SV, seminal vesicle.
U, uterus.
V, vitelline follicle.
VD, vitelline duct.
VE, vas efferens.
VR, vitelline reservoir.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GP'
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AC
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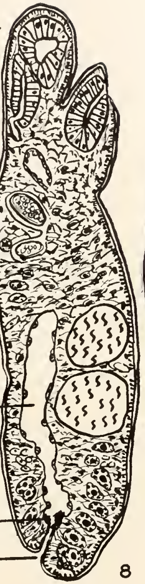
VD
PC

7



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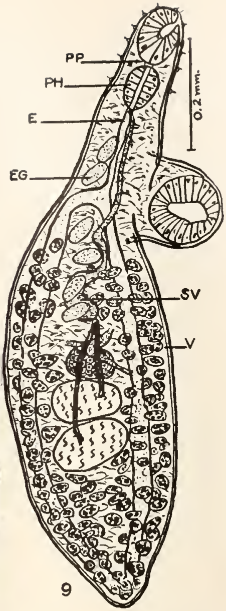
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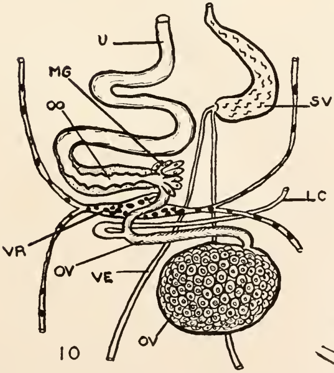
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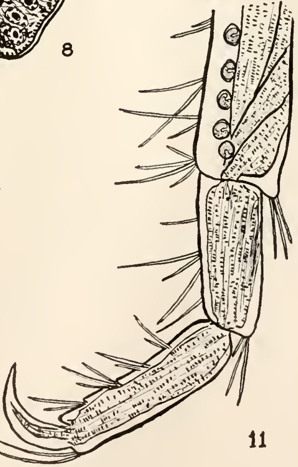

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PLATE II

size of body, suckers, testes, ovary and eggs, and the position of the genital pore. *A. eucinostomi* and *A. manteri* are similar in all respects except egg size and shape of the ovary. All three species differ widely in respect to host and locality.

Metacercaria (Figs. 3 and 5)

Metacercariae occur in the haemocoele throughout the body of the marine amphipods, *Carinogammarus mucronatus* (Say) (Fig. 4) and *Amphithoë longimana* Smith. Of 239 amphipods (143 *C. mucronatus* and 96 *A. longimana*) examined for natural infections, 10 per cent were positive. The number of cysts per amphipod varied from one to seven, averaging between two and three.

TABLE I

Comparison of Species of Anisoporus

Species	*A. cobraeformis* Ozaki, 1928	*A. eucinostomi* Manter, 1940	*A. manteri* Hunninen and Cable, 1940
Length (mm.)	4.3–7.2	1.222–2.497	0.74–2.32
Width (mm.)	0.33–.5	0.345–.465	0.28–.46
Oral sucker			
width (mm.)	0.16–.21	0.109–.144	0.07–.14
Ventral sucker			
width (mm.)	0.2–.28	——	0.12–.17
Ovary			
width (mm.)	0.13–.23	——	0.09–.14
shape	globular	subtriangular	ovoid
position	separated from anterior testis	close to anterior testis	close to anterior testis
Testes			
width (mm.)	0.16–.33	——	0.14–.2
Eggs			
length	0.044–.046	0.041–.048	0.062–.068
width	0.03–.033	0.025–.029	0.035–.04
Genital pore			
position	closer to ventral than to oral sucker	very close to pharynx	very close to pharynx
Locality	Japan	Galapagos Is.	Massachusetts, U. S. A.

The encysted worm is folded on itself (Fig. 3) and closely surrounded by an elastic cyst membrane 0.004 mm. thick. The membrane is easily ruptured with a needle. The size and shape of the metacercaria depend on the age of the infection, young cysts being spherical and less than 0.15 mm. in diameter; older ones are ovoid and measure as much as 0.785 by 0.675 mm. In large cysts the worms become sexually mature and eggs are laid within the cyst. One metacercaria contained 24 eggs, most of which were free in the cyst fluid. These eggs measured 0.061–.66 (av. 0.064) mm. in length by 0.035–.39 (0.038) mm. in width and appeared to be as normal as those in worms removed from the definitive host.

Amphipods were easy to infect experimentally. In one group of 11 amphipods exposed for four days to cercariae, one was negative on examination while ten contained 4, 4, 7, 7, 8, 8, 13, 13, 15 and 17 small metacercariae, all of about the same size. In another experiment, 11 amphipods were exposed to cercariae for five days and found upon examination to contain 1, 1, 6, 7, 7, 10, 10, 12, 14, 28, and 334 cysts. Two amphipods in this group were naturally infected, one with two cysts and the other with one, all three being considerably larger than the metacercariae from experimental infections of the same amphipods. Exposure of amphipods to large numbers of cercariae results in heavy infections in which the cysts are found throughout the body, even in the antennae and legs (Figs. 4, 11).

In 2-day-old metacercariae (Fig. 3), the stylet is still present but is absorbed between the third and fourth days. Soon after encystment, the relatively long prepharynx of the cercaria begins to shorten so that in older metacercariae it is visible only when the body is extended. Dobrovolny (1939*b*) has described a similar shortening of the prepharynx in *Plagioporus lepomis* during post-cercarial development.

Cercaria (Figs. 1–2)

Specific Diagnosis.—Modified cotylomicrocercous type. Body contracted 0.12 long, extended over 0.325, average 0.225; cuticula aspinose; oral and ventral suckers lined with fine spines; a circlet of about 10 papillae with sensory "hairs" surrounding mouth. Tail extended 0.048–.056 long and 0.018 wide near base, moderately contracted 0.028–.039 long and 0.031 wide, filled with glands the ducts of which form a protrusible papilla. Oral sucker 0.035 long and 0.031 wide; stylet double pointed, 0.01–.011 long and 0.005 wide; prepharynx long and slender, pharynx 0.011 in diameter. Ventral sucker 0.032 in diameter. Three pairs of cephalic glands with a single lateral and two median ducts on each side. Oval excretory vesicle filled with granular masses. Main excretory tubules ciliated, dividing at level of acetabulum to form anterior and posterior collecting tubules. Excretory formula $2[(2+2)+(2+2)]$. Develop in sausage-shaped sporocysts with terminal birth pore.

Host.—*Mitrella lunata* (Say).

Locality.—Waquoit Bay, Cape Cod, Massachusetts, U. S. A.

The cercaria of *A. manteri* differs from described cotylomicrocercous cercariae in that the tail, instead of forming a hollow sucking cup, is filled with large gland cells which secrete a sticky substance. The tail becomes so firmly attached to objects that the cercaria is not dislodged by water currents of considerable force. In addition to extension and contraction of the body during the waving, exploratory, and inch-worm movements characteristic of most cotylomicrocercous cercariae, the larvae of *A. manteri* have a peculiar type of behavior, commonly observed in attached specimens. The extended cercaria loops on itself so that its shape is approximately that of the letter " e " written vertically. The body is then straightened with a sudden spiral movement. This coiling and uncoiling movement sometimes gives the illusion that the cercaria reverses ends.

Sporocysts of *A. manteri* occur in the branchial region and digestive gland of the snail. They are simple, sausage-shaped forms with a protrusible anterior end bearing the birth pore. The largest sporocyst measured was 0.65 mm. long and contained 35 apparently mature cercariae.

Discussion

The present study demonstrates that *Anisoporus* and probably the related genera *Opecoelus, Opecoeloides,* and *Opegaster* are co-familial with other trematodes having cotylomicrocercous cercariae and do not constitute a distinct family as maintained by Ozaki (1925, 1929). Hence, Odhner (1928) and Stunkard (1931) are supported in their opinion that anal openings in these trematodes are of significance only in the separation of genera and species.

If, in the phylogeny of the trematodes, convergent evolution has occurred, one would expect to find evidences of it in the parasites of fishes, the oldest class of vertebrates. Convergent evolution would account for similarity of adult stages of species having fundamentally different larvae as now well demonstrated in the Allocreadiidae. On the other hand, divergent evolution apparently has led to the separation of adults which appear to be distantly related but actually have similar larval stages. An excellent illustration is afforded by the separation of the Acanthocolpidae from the Allocreadiidae on the basis of spines in the cirrus and metraterm. According to Martin (1939), the acanthocolpid, *Stephanostomum tenue,* has an ophthalmoxiphidiocercaria while the authors (Cable and Hunninen, 1940) have found that another acanthocolpid, *Deropristis inflata,* has a trichocercous larva. Not only are these larvae dissimilar, but each displays characteristics common to certain members of the family Allocreadiidae. Hence a classification based on actual relationships will require the combination and reclassification of the Acanthocolpidae, Allocreadiidae, and possibly the Monorchiidae.

The task of reclassifying the group would be a formidable one and must evaluate both larval and adult characters with great care. Larval and particularly cercarial structures may actually be misleading in some cases, particularly in groups in which only a few life histories are known. The elimination of a free-swimming period during cercarial life is often accompanied by extreme reduction and even complete loss of the tail; such modification may occur even in cercariae which emerge from aquatic hosts. A good illustration is afforded by the brachylaemids whose cercariae were known until very recently only from pulmonate gastropods, many of which are terrestrial. In these hosts the tails of the cercariae are extremely rudimentary or lacking altogether. Recently, however, Allison (1940) has found in the prosobranch snail, *Campeloma,* a furcocercous brachylaemid cercaria which, as he states, strongly suggests a relationship between the Brachylaemidae and other trematodes having furcocercous cercariae.

When considered only as formulae, excretory systems also may be misleading, as exemplified by *Cercaria coronanda* Rothschild, 1938, the cercaria of *Exorchis oviformis* as described by Komiya and Tajimi (1940), and an undescribed species discovered by the authors, all of which are true pleurolophocercous cercariae but with an excretory formula of $2[(2+2)+(2+2)]$. This formula is the same as that of the microphallids but a consideration of other larval characteristics gives no reason to consider the cercariae mentioned as intermediate types between the pleurolophocercous larvae of the Opisthorchioidea and the xiphidiocercous larvae of the Microphallidae. It is clear that the excretory pattern must be correlated with other larval characteristics, all of which must be considered as a whole.

There are pleurolophocercous cercariae without eye-spots, cotylomicrocercous forms without stylets, microphallid larvae without tails, and strigeid fork-tails without pharynges. Yet other larval characteristics give definite clues to relationships.

Much has been accomplished by the study of adult material as indicated by the manner in which life-history studies have confirmed relationships postulated on the basis of adult morphology. Structure of adults may be the deciding factor in the classification of species having cercariae so modified that they might be considered either as aberrant members of some well-defined group or as a distinct larval type. For example, the cercaria of *Monorcheides cumingiae* (Martin, 1938) differs from typical trichocercous species in the nature of the tail and excretory pattern and may be a separate larval type. Determining the relationship of this species to other trematodes, and hence the validity of the family Monorchiidae, may depend as much on adult as larval characters, particularly until more than one life cycle in the family is known.

It is concluded that a revision of the Allocreadiidae is needed and at present could be proposed to the extent of defining families and placing certain genera in them. Even so, our knowledge of the morphology and life histories of many genera is so incomplete that their allocation to families would be a matter of conjecture.

We believe that the trematodes at present assigned to the family Allocreadiidae represent at least three distinct families, possibly belonging to more than one superfamily. This belief is justified if the heterophyids and opisthorchiids, with practically identical cercariae, are correctly regarded as separate families of the same superfamily.

Very recently, Hopkins (1941) has accepted the validity of the family Opecoelidae and included in it all allocreadoid genera having an excretory formula of $2[(2+2)+(2+2)]$, and cercariae of the cotylomicrocercous type. Leaving all other genera in the family Allocreadiidae, he includes in the Opecoelidae the genera *Cymbephallus, Podocotyloides, Enenterum, Dactylosomum, Coitocaecum, Genitocotyle, Nicolla, Ozakia,* as well as the more typical opecoelid genera *Opecoelus, Opegaster, Opecoeloides, Anisoporus,* and *Opecoelina.* Hopkins also implies but does not definitely state that *Helicometra, Plagioporus, Hamacreadium, Sphaerostoma,* and *Podocotyle* also should be included. Since Mathias (1937) has found that *Allocreadium angusticolle* has a cotylomicrocercous cercaria, the genus *Allocreadium* also should be included in the above group. Obviously, the name Allocreadiidae is available only for the family including the type genus *Allocreadium;* this genus must be regarded as co-familial with the genera Hopkins allocates to the family Opecoelidae. Either the name Allocreadiidae or Opecoelidae must be suppressed. Since Allocreadiidae is an older and more familiar name than Opecoelidae and Ozaki proposed the family Opecoelidae without knowledge of either excretory systems or life histories, but separated it from the Allocreadiidae on the basis of characters which have not been generally accepted as valid, it is proposed that the name Allocreadiidae take precedence over Opecoelidae. This proposal simply means that the family Allocreadiidae is restricted to include only those forms having cotylomicrocercous cercariae and a simplified excretory pattern. In any event, it will be necessary to propose new families or redefine existing ones to include the genera excluded from the restricted family. Such a revision is beyond the scope of the present paper.

SUMMARY

The life history of *Anisoporus manteri* Hunninen and Cable, 1940, has been traced experimentally. The cercaria, a cotylomicrocercous type,

develops in sporocysts in the marine snail, *Mitrella lunata* (Say) and encysts in the marine amphipods, *Carinogammarus mucronatus* (Say) and *Amphithoë longimana* Smith. Old metacercariae contain eggs in the uterus and cystic fluid. Adult worms occur in the intestine of the marine fishes, *Syngnathus fuscus* Storer, *Paralichthys dentatus* (Linnaeus), *Hippoglossoides platessoides* (Fabricius), *Apeltes quadracus* (Mitchill), *Fundulus heteroclitus* (Linnaeus) and *F. majalis* (Walbaum).

It is proposed that the family Allocreadiidae be restricted to include only trematodes having cotylomicrocercous cercariae and simplified excretory patterns, since the type genus, *Allocreadium,* would be included in the restricted family. Consequently, the family name, Opecoelidae, would be suppressed as a synonym of Allocreadiidae *sensu stricto.*

Addendum and Correction

In respect to the uroproct, the present species is more like *Opecoeloides* than *Anisoporus.* Odhner (1928) erected the genus *Opecoeloides* to contain a single species, *Distomum furcatum* Bremser, and described in this form the union of the ceca with the excretory vesicle as has been observed in the present study. In *Anisoporus,* as defined by Ozaki (1928), instead of joining the excretory vesicle, the ceca unite posteriorly to form a median tube with an anal opening independent of the excretory pore. In other respects, the genera *Opecoeloides* and *Anisoporus* are identical. While the present paper was in preparation, the writers were inclined to share with Manter (1940) doubt as to the generic significance of the uroproct as well as sucker papillae, characters used extensively in the separation of genera. The paper in which Odhner proposed the genus *Opecoeloides* appeared Nov. 13, 1928, while Ozaki's definition of *Anisoporus* was published Dec. 31 of the same year. Whether or not these genera are regarded as synonymous, *Opecoeloides* is the older name and the present species must be placed in that genus; accordingly, the correct designation is *Opecoeloides manteri* (Hunninen and Cable, 1940). The species for which Odhner erected the genus has never been mentioned in the literature except as *Distomum furcatum;* its proper designation is *Opecoeloides furcatum* (Bremser in Rudolphi, 1819). The writers have been unable to find a satisfactory description of this species, but from Odhner's paper it is possible to differentiate *O. furcatum* and *O. manteri,* the only species in the genus, on the basis of sucker papillae; there are six in *O. furcatum* and five in *O. manteri,* three anterior and two posterior. Since the present study supports the observations of Odhner and adds a second species of *Opecoeloides,* the validity of the genus is strengthened considerably. It therefore seems advisable to maintain *Anisoporus* and *Opecoeloides* as distinct genera, particularly since proposing synonymy for them would cause a certain amount of confusion.

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THE BLOOD OF THE ATLANTIC SALMON DURING MIGRATION

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There are many alterations in the habits and metabolism of salmon during their migration from the sea into the rivers. Among the metabolic changes, one can be well defined in relation to an equally distinct alteration of the environment of the fish. In fresh water the freezing point depression of the blood of salmon is less than it is while they are in salt water (Greene, 1904; Smith, 1932). As a consequence of the reduced salinity of the blood, which is indicated by the reduction of the freezing point depression, it might be expected that the condition of the blood for the transport of oxygen would be affected; for it is known that the affinity of hemoglobin in solution for oxygen is diminished by increasing concentrations of salt (Barcroft and Camis, 1909).

The affinity of the hemoglobin in the blood of several freshwater fish for oxygen is greater than in the blood of some saltwater fish, as is shown by the pressure of oxygen required for half saturation of the blood of a few marine and freshwater species in Table I. The freezing point depression of the blood of freshwater fish is usually less than in saltwater fish, and the blood of fish migrating from salt into fresh water undergoes dilution, as is shown by the examples in Table II. The examples quoted are too few to warrant more than the suggestion of the effect of salinity upon the blood, and there are many influences beside the salinity of the environment which will operate to differentiate the blood of various species.

There is an advantage in examining the blood of individuals of the same species in two environments separated by so short an interval as that which comes between the salmon in the brackish estuary and in the lower fresh water reaches of a stream. We have found it possible to distinguish the conditions for oxygen combination with the blood of Atlantic salmon, *Salmo salar,* caught in salt water from those of the fish which were caught in the rivers. The changes in oxygen affinity occurred as the freezing point was changed by the passage of the fish into fresh water. The changes observed are large enough to suit the blood for respiratory transport under somewhat different conditions, and may be critical in determining respiratory ability in certain natural situations.

MATERIALS AND METHODS

The blood of the Atlantic salmon, *Salmo salar,* was chosen because of its accessibility. These fish spawn and are hatched in the rivers which drain into the coastal waters of the Province of Quebec, Newfoundland, Nova Scotia, and New Brunswick. They spend the first two to five years of their lives in the rivers and then migrate to the ocean, where they spend one, two, or three years before returning to the rivers to spawn. The major spawning migrations occur in the spring and fall of the year, but fish in greater number and of larger size come in the spring (at least in the St. Lawrence region (Belding and Prefontaine, 1938)). Our samples of fish were obtained in the late spring and early summer from the gill nets of commercial fishermen situated around the mouth of the York River, which empties into Gaspé Bay, Province of Quebec. These fish were in brackish water and, unfortunately, no true saltwater fish were obtained. Freshwater fish were had from the lines of the sport fishermen on the St. Jean River, which also empties into the Bay. Two of the freshwater fish were kindly supplied to us from the salmon of the Gaspé Hatchery.

TABLE I

A comparison of the tensions of oxygen required for half saturation of the blood of some salt and freshwater fish

Fish	Tension at half saturation mm. O_2	pCO_2 mm.	Temperature °C.
Fresh water			
Bowfin * *Amia calva*	4	0–1	15
Common sucker * *Catostomus commersonnii*	12	0–1	15
Carp * *Carpiodes cyprinus*	5	1–2	15
Pike † *Esox lucius*	3.5	7.5	18
Salt water			
Cod † *Gadus callarias*	15	7.5	14
Sea robin ‡ *Prionotus carolinus*	16	1	20
Mackerel ‡ *Scomber scombrus*	16–17	1	20
Toadfish ‡ *Opsanus tau*	14	1	20

* Black.
† Krogh and Leitch, 1919.
‡ Root, 1931.

The fish were all two- and three-year "sea life" salmon averaging roughly 10 and 20 pounds in weight respectively. They were bled by heart puncture, 20 to 80 cc. being obtained from a single fish. Heparin was used as the anticoagulant throughout, and the blood was stored on ice from the time of drawing until it was used.

The blood was equilibrated with gas mixtures in the special tonometers designed by Irving and Black (1937). Gas analyses on blood were done by Van Slyke's manometric method. The temperature of equilibration was 15° C. ± 1.0°.

Freezing point determinations were made with a micro-Beckmann thermometer on 2 cc. samples of plasma. Despite the small samples, most duplicate determinations agreed to within .005° C.

Hematocrit determinations of relative erythrocyte volume were made on all specimens in the usual capillary tubes in a centrifuge operating at 5000 r.p.m.

TABLE II

A comparison of the freezing point depressions of the blood of fresh, salt water and migratory fish. Modified from Smith (1930)

	Freezing Point °C.	
Fish	Medium	Fish plasma
Amia calva	.03	.54
Lepidosteus osteus	.03	.57
Anguilla rostrata	.08	.63
" "	1.85	.82
Conger vulgaris	2.15	1.03
Salmo salar	0	.64
" "	.87	.77

OXYGEN CAPACITY

The oxygen capacities (Table III) of the bloods of 15 brackish water fish varied between 10.5 and 14.9 volumes per cent, with an average capacity of 12.3 volumes per cent. The proportion of erythrocytes in the blood of these fish varied from a minimum of 24.4 per cent cells to a maximum of 47.5 per cent cells, averaging 39.4 per cent. In 6 freshwater fish the oxygen capacities varied between 6.7 and 10.0 volumes per cent with an average of 8.8; the proportion of erythrocytes ranged from 19.3 to 28.4 per cent, averaging 24.8. If we calculate the oxygen capacity of 100 cc. of cells for the two kinds of fish, we find that for the brackish water fish the average value is 31.6 cc., whereas for the freshwater group it is 34.9 cc. It seems from these figures that the proportion of cells in the blood of freshwater fish was decreased without diminution of the oxygen capacity of the cells.

OXYGEN DISSOCIATION CURVES

Points on the oxygen dissociation curve of the brackish water fish show a considerable scatter. The points are taken from 14 different fish. It can be seen from Fig. 1 that at half saturation the spread is from about 17.5 to 28.8 mm. of O_2 tension. The mean curve has a half saturation at 23 mm. O_2 tension. The curve is similar to those found for other marine fish by Root (1931). The curve shows an interesting tendency to have an " S " shape similar to that of mammalian bloods, and is in most other respects similar to these well-known curves.

In contrast to the brackish water fish, it is to be noted that with the exception of one point the points obtained from 4 freshwater fish fall nicely on a continuous curve. This curve is rather steeper than the others and lies to the extreme left of them with a half saturation at about 19 mm. of O_2 tension (Fig. 1).

TABLE III

A comparison of the data on brackish and fresh water salmon

	Brackish water fish	Fresh water fish
Cell volume	39.4%	24.8%
Variation	24.4–47.5 (15 fish)	19.3–28.4 (6 fish)
Oxygen capacity	12.3 vols. %	8.8 vols. %
Variation	10.5–14.9 (15 fish)	6.7–10.1 (6 fish)
Maximum CO_2 effect	62.8% Sat.	57.2% Sat.
Variation	56–67 (5 fish)	56–58 (4 fish)
Cell volume increase	9.0%	8.2%
Variation	8.1–13.9 (5 fish)	6.2–11.1 (4 fish)
Depression of freezing point	0.77° C.	0.64° C.
Variation	0.72–0.80 (5 fish)	0.60–0.68 (7 fish)

THE EFFECT OF CO_2 UPON OXYGENATION

Carbon dioxide prevents the saturation of salmon blood with oxygen at 150 mm. oxygenation (Fig. 2). This has been observed in the blood of other marine and freshwater fish (Root, 1931; Black and Irving, 1938; Irving, Black and Safford, 1941).

There is a considerable spread in the points obtained from the brackish water fish, the maximum effect of CO_2 restricting the oxygenation of hemoglobin to between 56 and 67 per cent saturation. In the freshwater fish CO_2 restricted oxygenation to about 58 per cent saturation for the maximum effect. Neither curve for salmon blood seems to flatten out quite as quickly as the curve of carp blood (Black and Irving, 1938), and even beyond pressures of 80 mm. of CO_2 there appears to be some further depression of oxygenation. Hemolysis does not abolish the effect of CO_2 either in the brackish or freshwater fish, and causes a decrease of not over 15 per cent in its magnitude. Hemolysis of trout blood likewise does not much reduce the effect of CO_2 upon oxygenation (Irving, Black and Safford, 1941). There are evidently two categories of fish blood, in one of which hemolysis abolishes the CO_2 effect (Black and Irving, 1938; Root and Irving, 1940), while in the other hemolysis has little influence. Hemolysis by either freezing and thawing or by the use of saponin produces the same result.

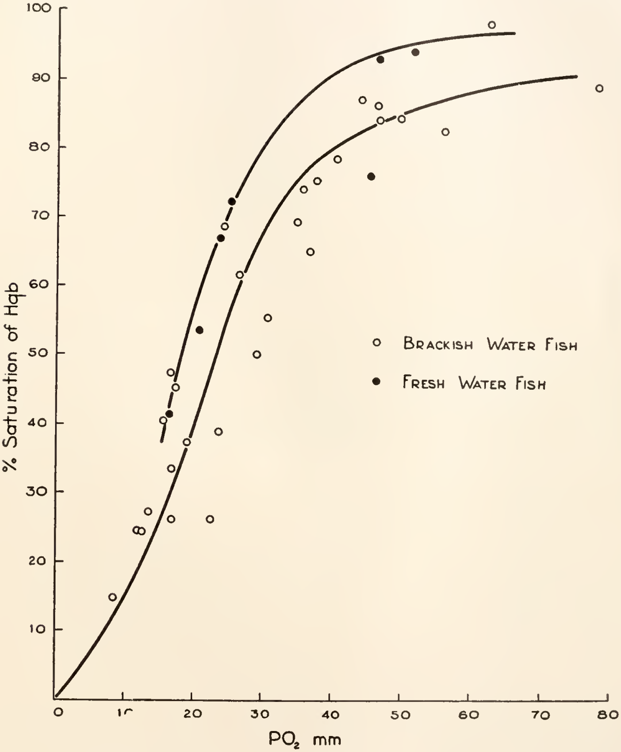

Fig. 1. The O_2 dissociation curve of the blood of salmon taken from brackish and fresh water. P CO_2 less than 1 mm.

The effect of CO_2 on the oxygen dissociation curve of brackish water fish is shown in Fig. 3. The pressure at half saturation of the hemoglobin is 40 mm. of oxygen when the CO_2 tension is 13–14 mm. compared with the average of 23 mm. O_2 tension with the CO_2 tension of 1 mm. or less. The effect of CO_2 upon the oxygen dissociation curve of freshwater salmon was not determined, but from the maximum effect of CO_2 on the oxygen capacity in fresh and saltwater fish it seems likely that the effect of CO_2 on oxygen dissociation curves would be similar in salmon from salt and fresh water.

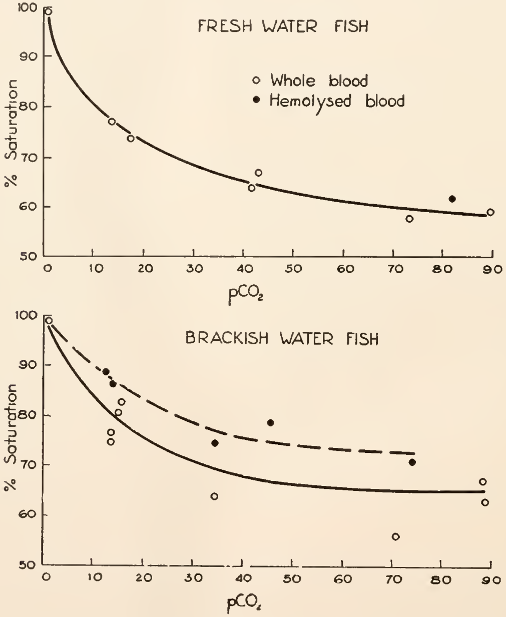

FIG. 2. The effect of CO_2 upon oxygen dissociation curves of salmon blood.

EFFECT OF CO_2 ON CELL VOLUME

Carbon dioxide has a marked effect in causing the cells of salmon blood to swell (Fig. 4), as was shown for the blood of some freshwater fish by Black and Irving (1938), and for the blood of trout (Irving, Black and Safford, 1941). The magnitude of the effect on the cell volume in brackish water fish averaged 9.0 volumes per cent of cells, with a variation of 5.1 to 13.9 volumes per cent in 5 fish. In the freshwater fish the swelling averaged 8.2 with a variation of 6.2 to 11.1 in 4 fish. The reversal of the effect on complete oxygenation of the blood without CO_2 is demonstrable.

Most of the swelling is produced at low tensions of CO_2, and it indicates some 25 per cent enlargement of the erythrocytes. The considerable swelling of the cells is of a much greater magnitude than the osmotic changes which are described in mammalian blood, and there seems to be no satisfactory explanation for this phenomenon.

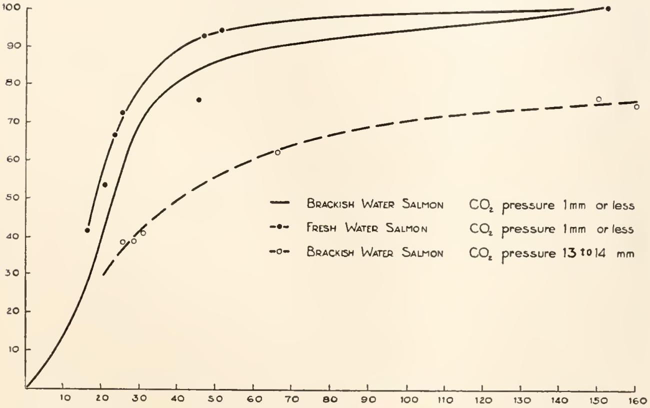

FIG. 3. The maximum effect of CO_2 upon the degree of oxygen saturation at P O_2 = 150 mm.

THE COMBINATION OF CO_2 WITH BLOOD

In Figure 5 is shown the curve describing the combination of CO_2 with the blood. This curve indicates that in contrast to the reduction in oxygen capacity which occurs in fresh water, there is no significant difference in CO_2 combination.

FREEZING POINT OF THE PLASMA

The effect of the change of external environment upon the blood plasma is demonstrated in the change in the freezing point depression. In the plasma of brackish water fish freezing points varied from —0.717

to — 0.800° C., with an average value of — 0.765° C. for the 5 bloods examined. In the freshwater fish 7 determinations fell between — 0.597 and — 0.675° C., with an average of — 0.638° C. One other fish showed a value of — 0.775° C. This fish was caught much lower down in the river than any of the others, although still in the fresh water. It was caught at the very end of the season when it is known that the

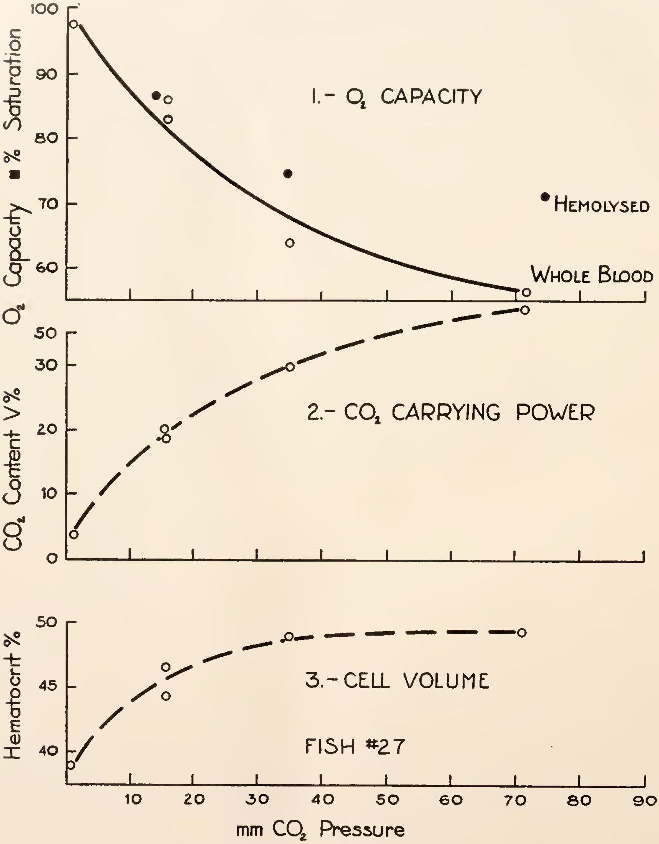

FIG. 4. The effect of CO_2 upon: (1) oxygen saturation, (2) CO_2-carrying power, and (3) cell volume in the blood of one salmon.

late stragglers spend very little time in becoming acclimated to the brackish water, and this observation may explain the individual discrepancy.

DISCUSSION

We would like primarily to answer the question in this paper of whether or not there is a difference in the properties of the blood of a migratory fish when it is in equilibrium with a marine and freshwater environment, and the changes which we are particularly interested in are those of the system for respiratory transport.

There is undoubtedly a drop in the concentration of cells of the salmon in fresh water, and concomitantly there is a drop in the oxygen capacity of the blood (Table III). There appears to be no difference in the hemoglobin concentration of the cells of the two kinds of fish.

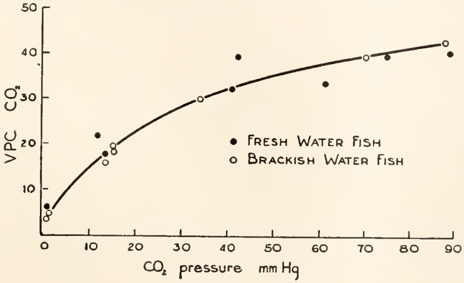

FIG. 5. The combination of CO_2 with salmon blood at P $O_2 = 150$ mm.

Dilution of the serum also takes place when the fish moves into fresh water, as shown by the decrease in the freezing point depression. If we examine the oxygen dissociation curves of the fish in its different habitats, there appears evidence that here too there is a change with migration. The curve for the freshwater salmon lies on the outermost border of the scatter of points belonging to the brackish water inhabitants (Fig. 1), and the points hold well to a smooth curve.

Considering the brackish water fish in relation to their environment, we find: (1) that the environment varied somewhat in its osmotic concentration with respect to the tide and the location in the bay in which they were caught; (2) that the fish had been in this environment of lower salinity than the sea [1] for various lengths of time and were consequently in various stages of acclimitization. In view of this we might expect to find the variation which was actually observed, and also to predict that the blood of true marine fish should give points lying at the extreme right of the group. We are inclined then to believe that in this important characteristic, the affinity for oxygen, the blood of salmon living in fresh water differs from those taken from salt water.

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[1] The freezing point depression of a sample of water from the mouth of the York River, at which point many of the fish were taken, was 0.87.

The provision for transporting oxygen in fresh water is less since the oxygen capacity of blood is only about two-thirds of what it is in the brackish water, and probably less than that in relation to the oxygen capacity of true marine fish. One is inclined to wonder if the fresh-water environment imposes a more sedentary mode of life because of the changes which are brought about in the internal conditions of the salmon.

In affinity for oxygen the blood of fresh water salmon equals that of three freshwater salmonoid fish,—the brook trout, brown trout, and rainbow trout (Irving, Black, and Safford, 1941). In all of these fish atmospheric tensions of oxygen saturate the hemoglobin at 15°. But as the temperature is raised, the affinity of trout blood was found to diminish, until at about 25° atmospheric pressures of oxygen could no longer secure saturation. It is quite likely that a similar temperature effect prevails in salmon blood, which should therefore be suitable for oxygenation at 25° in thoroughly aerated water. The blood of salt-water salmon would probably fail to saturate at about 20°, but it is likely that the well-circulated tidal waters are always well enough aerated for adequate oxygenation. In the warm water of the rivers in summer even the blood of the freshwater salmon encounters temperatures which are near the limit permitting saturation. If the oxygen tension in the warm water is depleted below atmospheric pressure, then the blood cannot become saturated with oxygen in the gills. In the rivers it is likely that stretches of warm water, partcularly if they are not well aerated, act as barriers by hindering the transport of oxygen. Under such conditions the effect of temperature upon oxygen affinity may have a critical influence in determining where the fish can exist.

We see that the plasma of freshwater fish has a lower freezing point than the brackish water fish, and that this is due to a reduction in the electrolyte concentration has been shown by Homer Smith (1930). In our freshwater salmon, the serum has been diluted and we may surmise that in order to reestablish osmotic equilibrium, water has diffused into and perhaps salts out of the cells. That the latter is so would seem to be borne out by the values of oxygen carried by 100 cc. of cells. Long ago Barcroft and Camis (1909) showed that when hemoglobin solutions are dialyzed, the oxygen dissociation curve for the solution, when compared with the curve for the undialyzed solution, is shifted some to the left, and hence saturates at a lower pressure. It should be pointed out that they were working with rather dilute solutions of hemoglobin and also were dialyzing off the last portion of electrolyte and therefore the conditions in the salmon blood are hardly comparable.

It is rather remarkable that the CO_2 dissociation curves for the freshwater and saltwater fish (Fig. 5) should be the same in spite of the difference in oxygen capacity. There are large differences in CO_2 capacity of the blood of different species of fish which appear to be quite unrelated to oxygen capacity.

The changes which have been shown in the blood of migrating salmon are large enough to be important to the economy of respiratory metabolism. The relation of these changes in the blood to the change in environment suggests how the detailed physiology of the salmon changes with the varying environment.

Summary

The blood of Atlantic salmon caught in the brackish water of Gaspé Bay has been compared with the blood of salmon caught in the fresh water of the rivers draining into the Bay. In brackish and fresh water the average properties of the blood are respectively: oxygen capacity, 12.3 and 8.8 volumes per cent; cell volume, 39.4 and 24.8 per cent; oxygen tension for half saturation at $T\ CO_2 = 1$ mm., 23 and 19 mm.; freezing point of the serum, —0.79 and —0.64. The oxygen combination at $P\ O_2 = 150$ mm. in the presence of large tensions of CO_2 is reduced to about 60 per cent of saturation. Hemolysis does not much reduce the CO_2 effect. The cells swell greatly as the CO_2 tension is increased. There appears to be a dilution of the blood as the fish goes from salt to fresh water. This is seen in the decrease in cell volume, oxygen capacity, and freezing point depression of the blood. It seems also that in fresh water the affinity of the hemoglobin for oxygen is greater than in salt water. The changes observed in the blood may be related to the change in salinity of the environment. In the warm water of rivers in summer small changes in temperature and oxygen saturation may be critical in determining whether or not the blood can be saturated with oxygen.

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COMPARATIVE STUDIES OF THE PIGMENTS OF SOME PACIFIC COAST ECHINODERMS[1]

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From the standpoint of comparative biochemistry, the echinoderms represent an interesting and little-explored phylum. Prominent among the biochemical features of this group is the conspicuous manifestation of body-pigments, striking in their intensity and color-varieties, and rivalling the beauty of those displayed by the sessile coelenterates.

The integumentary colors of sea-stars and brittle stars are due preponderantly to carotenoids, while certain of the echinoids manifest instead considerable quantities of pigments of the echinochrome class, first reported by MacMunn (1883*a*) and recently shown by Kuhn and Wallenfels (1939) to be naphthoquinones. Kuroda and Ohshima (1940) have crystallized three distinct spinochromes, each from a different species of Japanese sea-urchin, and have found the natural pigments to be very similar to synthetic hydroxynaphthoquinones. Some echinoids yield echinenone, a unique carotenoid which is a provitamin A (Lederer and Moore, 1936). Long ago, MacMunn (1883*b*, 1886) reported the presence of " enterochlorophyll " in the alimentary organs of a number of carnivorous echinoderms.

Studies of Abeloos (1926) and Lönnberg (1931, 1932, 1933) give qualitative indications of the nature and distribution of carotenoids in echinoderms. Euler and Hellström (1934) and Euler, Hellström and Klussman (1934) made chemical studies of the carotenoid proteins of asteroids, and isolated a new pigment, asteric acid. Karrer and Benz (1934) and Karrer and Solmssen (1935) isolated astacene from both an ophiuran and an asteroid. Lederer (1938) has studied the pigments of the echinoid *Strongylocentrotus lividus*.

Numerous writers emphasize the importance of the question as to whether some of the lower animals may be able to synthesize specific carotenoids from simpler molecules. Among the various ecological factors and physiological activities which may influence the pigmentation of animals, it is probable that food exerts the closest and most direct effect, although habitat and various physiological adaptations inseparably associated with nutrition are necessarily important in a survey such as we have undertaken.

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Of the rather scanty information available regarding the physiology of adult echinoderms, facts concerning their feeding mechanisms and digestive enzymes are probably the chief entries. Yonge (1928) discusses the diversity of feeding mechanisms employed by the Echinodermata, and divides the phylum into two main groups as regards feeding and digestion, namely: (1) the Asteroidea and Ophiuroidea which are exclusively carnivorous, and (2) the Echinoidea and Holothuroidea, chiefly herbivorous and to some extent omnivorous. He suggests (1931) that the ciliary-feeding Crinoidea may be primarily herbivorous.

In the present study, we have attempted to make a preliminary classification of the various echinoderms into biochemical (pigment) types, with correlative differences in intraphylar class, sex (where practicable), habitat and nutritional habits occupying collateral positions of importance.

The survey, which included four species of three genera of echinoids, one species of holothuroid, four species of three genera of asteroids, and three species of two genera of ophiuroids, revealed numerous familiar and a few new carotenoids. Purple echinochrome pigments were observed only in echinoids. Small amounts of green " enterochlorophylls " were found among the asteroids and ophiurans. We have considered the echinoderm classes in ascending evolutionary series within the two chief nutritional groups named by Yonge (1931) i.e. (1) herbivore-omnivores: (a) echinoids, (b) holothuroids; and (2) carnivores: (a) asteroids, (b) ophiurans. Consistencies in this classification will be apparent in Table I.

The animals were collected at several localities in this vicinity of the Southern California coast, and in some cases obtained by dredge hauls. We are indebted to Mr. P. S. Barnhart, curator, Dr. C. E. Moritz, visitor from Dartmouth College, Professor G. E. MacGinitie of the California Institute of Technology, Mr. Granville Ashcraft of the Hancock Foundation, University of Southern California, Professor H. J. van Cleave, visitor from the University of Illinois, and Mr. L. D. Pratt of the Kelco Company, National City, for much of the material used. Mr. Sheldon C. Crane rendered technical assistance during the latter part of our carotenoid investigations.

Methods

With but few exceptions noted below, the animals were placed temporarily in running sea water in laboratory aquaria, to permit foreign material to be evacuated from the intestine. The carotenoid pigments were extracted from the ground tissues with acetone, passed into petroleum ether, subjected to partition between the latter and 90 per cent methanol, and separated into individual components by chromatographic adsorption, in accordance with regular procedures described in more detail by Scheer (1940) and by Fox and Pantin (1941).

Individual pigments were provisionally identified by comparison of certain of their properties with those of known pigments; this identification must wait upon further studies for full confirmation. The following properties were used: (1) Behavior in partition between immiscible solvents, i.e., petroleum ether (which constitutes the epiphase and dissolves carotenes, xanthophyll esters and a few monohydroxyxanthophylls) and 90 per cent methanol (the hypophase which selectively removes xanthophylls). (2) Adsorption behavior on Tswett chromatographic columns, xanthrophylls and their common esters being adsorbed from benzene solution on calcium carbonate, while carotenes pass through this but are selectively adsorbed on calcium hydroxide (Zechmeister and Cholnoky, 1937; Strain, 1938). (3) Positions of spectral absorption maxima. (4) Behavior toward the partition test after treatment with hot alcoholic potassium hydroxide in an inert atmosphere; carotenes remain unchanged; xanthophylls, epiphasic when esterified, are rendered hypophasic when hydrolyzed; astaxanthin, free or esterified, neutral before treatment, is transformed into astacene, with definite acidic properties.

Positions of absorption maxima were determined with a Hartridge reversion spectroscope and with a Bausch and Lomb spectrophotometer. The two instruments show good agreement excepting in the case of acidic carotenoids like astacene, whose single broad maximum is more accurately determined with the latter instrument. Carbon disulphide was employed as the solvent unless otherwise specified.

Relative concentration of mixed carotenoid pigments (i.e., whole epiphasic or whole hypophasic fractions) were estimated in terms of "β-carotene equivalents," the extinction coefficient of β-carotene at 485 mμ being determined by other writers (Smith, 1936) and ourselves.

Echinochrome pigments were readily extractable from the echinoids by treatment of the whole tests with dilute hydrochloric acid under a layer of diethyl ether which readily dissolved the pigments (see Tyler, 1939). These were subsequently examined spectroscopically in ether or chloroform solutions.

Green pigments ("enterochlorophylls") were recovered in small amounts from the digestive diverticula of two of the asteroids, *Pisaster ochraceous* and *P. giganteus,* and from the whole-body extracts of all three ophiurans. The material recovered from the ophiurans differed in certain solubility properties from that yielded by the asteroids, but all showed closely agreeing absorption spectra. Actual quantities were so small that no identifying tests were practicable excepting those employed; hence the descriptive term coined by MacMunn has been used provisionally in the discussion of these green pigments.

RESULTS

A summary of the distribution of carotenoids, echinochromes and enterochlorophylls is given in Table I, followed by more detailed information regarding the separate pigments.

Carotenoids

The distribution, kinds, and some relative quantities of individual carotenoids encountered in the various species are summarized in Table II.

Echinochromes

These pigments, present exclusively in the echinoids, showed a few interesting variations in tissue distribution as well as in chemical and spectroscopic properties, as shown in the following outline:

Dendraster excentricus: Purple aggregates of the pigment were present in ectodermal and endodermal tissues lining the shell of this purple sand-dollar, while similar bodies in mature male and female gonad tissues and in anterior portions of the gut were red. The posterior part of the gut lacked echinochrome. The gelatinous egg-cases contained red echinochrome bodies.

The pigment was purple in neutral or alkaline media and red in acid (see also Lederer, 1940). It was readily soluble in aqueous acetone, giving clear filterable solutions, and was also extractable from dilute acid digests of the shell with diethyl ether (see Tyler, 1939).

The absorption maxima were as follows:

In acetone: 524, 490 mμ.

In chloroform: 533, 496, 465 mμ (*cf.* Kuhn and Wallenfels, 1939).

Strongylocentrotus franciscanus: Much purple pigment was yielded to acetone by treatment of this large purple-red urchin. The pigment decomposed with bleaching, however, before it was given any study.

Strongylocentrotus purpuratus: This purple urchin, like the sand-dollar *Dendraster,* showed many aggregates of echinochrome in the ectoderm (purple) as well as in endoderm, coelomic fluid and gut wall (red in all). Neither male nor female gonad tissues, however, contained any of the pigment.

It showed the following absorption maxima:

In water (neutral, colloidal): 526.5, 591 mμ.

In water (acidic): 497 mμ (single, diffuse band).

In chloroform: 525, 490.5 mμ (*cf.* Lederer and Glaser, 1938).

Lytechinus pictus: This pale urchin yielded far less of the purple pigment than did *Strongylocentrotus* or even *Dendraster.* Pigment aggregates were observable, however, in parts of the skin lining the shell, in the gut and in gonad tissues of both sexes.

The pigment, insoluble in acetone or water, readily bleached by alkali, and slightly soluble in dilute acid, gave the following spectral absorption bands:

In water (colloidal suspension): 526, 491 mμ.

In diethyl ether: 533, 497, 467 mμ.

The echinoids contained not only different quantities of echinochrome pigments, in the order *Strongylocentrotus* > *Dendraster* > *Lytechinus,* but the pigments were somewhat different chemically. Lederer (1940) lists the distribution of a number of pigments of the echinochrome class in several echinoid species, and in their separate body parts.

Green Pigments

The "enterochlorophylls," found only in the digestive diverticula of two of the five species of asteroids and in whole extracts of the three ophiuroids, showed certain chemical differences.

Asteroidea

Pisaster ochraceous: A small quantity of the pigment was recovered in acetone from the digestive diverticulum, being absent from stomach, skin or other parts. Completely insoluble in petroleum ether, it was readily soluble in alcohol, acetone and diethyl ether. Its non-acidic character was demonstrated by its extractability from diluted alkaline alcohol with ether. Dissolved in absolute ethanol, it manifested a single sharp absorption band in the red at 661 mμ.

Pisaster giganteus: The green pigment in this species, recovered in traces from the same tissues as in *P. ochraceous,* was similar in its chemical properties and also yielded a single absorption band in pure ethanol at 661 mμ.

Astropecten californicus and *Patiria miniata* failed to yield green pigments.

Ophiuroidea

Ophiopteris papillosa: Extracted with acetone and soluble also in diethyl ether, the pigment was dissolved readily in petroleum ether (un-

TABLE I

Summary of Pigments in Echinoderms Studied

Animal (number analyzed)	Tissue; weight grams	Total epiphasic carotenoids mg./100 g.	Total hypophasic carotenoids mg./100 g.	Ratio; hypophasic/epiphasic	Carotenes	Xanthophylls	Xanthophyll esters	Acidic carotenoids or their precursors	Predominating *types* of carotenoid	Echinochromes	"Enterochlorophylls"
HERBIVORES; OMNIVORES											
ECHINOIDEA											
Dendraster excentricus											
(3 ♀)	whole; 31	0.052	0.011	0.211	++	+	−	. . .	β-carotene	+	−
(2 ♂)	whole; 20	0.105	0.038	0.362							
Strongylocentrotus franciscanus											
(1 ♂)	whole; 298	0.170	0.025	0.147	++*	+	−	. . .	β-carotene	+	−
S. purpuratus											
(3 ♀)	skin . . .	traces	traces	. . .			± (varying)	−	zeaxanthin, β-carotene.	+	−
	intestine; 5.67	2.99	6.04	2.02	++*	++					
	gonad; 8.64	2.00	0	0	++*	−					
(3 ♂)	skin . . .	traces	traces	. . .							
	intestine; 4.72	2.34	5.65	2.41	++*	++					
	gonad; 11.9	0.690	0	0	++*	−					
Lytechinus pictus											
(26 ♀)	whole; 131	0.493	0.351	0.712	++*	+	−	. . .	α- and β-carotenes, echinenone.	+	−
(13 ♂)	whole; 67	0.871	0.430	0.493							
(9 ♀)	intestine; 0.9	4.48	4.18	0.934	++	+					
	gonad; 2.6	0.862	0.283	0.328	++	+					
	test; 27.0	0.009	0.012	1.33	traces	traces					
(11 ♂)	intestine; 1.6	3.38	1.52	0.450	++	+					
	gonad; 5.3	4.48	0.528	0.118	++	+					
	test; 47.0	0.012	0.013	1.18	traces	traces					
HOLOTHUROIDEA											
Stichopus californicus											
(3)	whole; 127	0.013	0.010	0.769	+*	+	−	. . .	echinenone, pectenoxanthin.	−	−
(1)	whole; 58.0 (eviscerated)	0.015	0.021	1.40							

Table I—*Continued*

Animal (number analyzed)	Tissue; weight grams	Total epiphasic carotenoids mg./100 g.	Total hypophasic carotenoids mg./100 g.	Ratio; $\frac{\text{hypophasic}}{\text{epiphasic}}$	Carotenes	Xanthophylls	Xanthophyll esters	Acidic carotenoids or their precursors	Predominating *types* of carotenoid	Echinochromes	"Enterochlorophylls"
CARNIVORES											
ASTEROIDEA											
Astropecten californicus (17)	whole; 70.0	0.044	0.718	16.3	+*	+++	−	+	pectenoxanthin, astaxanthin.	−	−
Patiria miniata Color varieties (*a*) ochraceous buff, carmine, Indian purple (1)	whole; 176	0.028	0.434	15.5	+	+++	−	+	zeaxanthin, astaxanthin.	−	−
(*b*) scarlet, scarlet orange (1)	whole; 195	0.079	1.29	16.3	+	+++	−	+	zeaxanthin, astaxanthin.	−	−
Pisaster ochraceous Color varieties (*a*) Rufous (1)	2 rays+skin; 114	0.022	0.204	9.28	+	+++	−	+	zeaxanthin, astaxanthin, mytiloxanthin.		−
	pyloric caeca; 52	0.667	12.9	19.3	+	+++	−	+		−	+
(*b*) Chocolate (1)	2 rays+skin; 85	0.024	0.284	11.8	+	+++	−	+			−
	pyloric caeca; 13	0.455	9.01	19.8	+	+++	−	+		−	+
Pisaster giganteus (1)	2 rays+skin; 62	0.067	0.902	13.4	+	+++	traces	+	zeaxanthin, astaxanthin or metridene.	−	−
	pyloric caeca; 10.4	0.401	1.37	3.41	+	++	traces	+			+
OPHIUROIDEA											
Ophiopteris papillosa (4)	whole; 9.81	0.489	2.90	5.95	−	+++	+	?	taraxanthin, pectenoxanthin.	−	+
(53)	whole; 102	0.254	decomp.	(hypo. > epi.)							
Ophiothrix spiculata (35)	whole; 18	1.60	decomp.	(hypo. > epi.)	−	+++	+	+	taraxanthin; new xanthophylls.	−	+
Ophiothrix rudis (18)	whole; 8.8	1.40	decomp.	(hypo. > epi.)	−	+++	+	+	taraxanthin; new xanthophylls	−	+

* and echinenone.

TABLE II

Summary of Carotenoids in Echinoderms Studied

Animal	Epiphasic fraction (carotenes, esters)				Hypophasic fraction (xanthophylls, acidic carotenoids)			
	Chromatogram:				Chromatogram:			
	Total number of zones	Descending order of *principal* zones	Absorption maxima mμ	Corresponding known pigments	Total number of zones	Descending order of *principal* zones	Absorption maxima mμ	Corresponding known pigments
Dendraster excentricus	—	—	521; 484 (whole fraction)	β-carotene	—	—	518; 483 (whole fraction)	zeaxanthin
Strongylocentrotus franciscanus	6	II III IV	525; 488 520; 484 520; 484	echinenone β-carotene β-carotene	3	I (II) (III)	510; 479 511; 478 (whole fraction)	lutein lutein (isomers)
Strongylocentrotus purpuratus	5 to 9	I II III IV V	506; 478 — — 490 518; 483 510.5; 479.5	lutein *ester* echinenone β-carotene α-carotene	3 to 6	I II III	511; 481 511; 481 to 484 515; 480 to 484	petaloxanthin or antheraxanthin? zeaxanthin
Lytechinus pictus	3 to 7	I II III IV	490 519; 484 } 519; 486 } 507; 477	echinenone β-carotene α-carotene	4 to 5	I II III	512; 482 511; 479 509; 477	petaloxanthin or antheraxanthin lutein
Stichopus parvimensis	5	(combined extract)	521; 489	echinenone	4	(combined extract)	521; 489	pectenoxanthin?
Astropecten californicus	4	IV	519; 485	β-carotene	5	I II III	510; 481 505 (hot KOH →acid) 520; 489	antheraxanthin astacene or metridene pectenoxanthin
Patiria miniata	3 to 6	(combined extract)	—; 482 to 485	β-carotene (?)	4 to 6	II III	507 to 509 (hot KOH →acid) 517 to 521; 483 to 484	astaxanthin → astacene zeaxanthin

TABLE II—*Continued*

Animal	Epiphasic fraction (carotenes, esters)				Hypophasic fraction (xanthophylls, acidic carotenoids)			
	Chromatogram:				Chromatogram:			
	Total number of zones	Descending order of *principal* zones	Absorption maxima mμ	Corresponding known pigments	Total number of zones	Descending order of *principal* zones	Absorption maxima mμ	Corresponding known pigments
Pisaster ochraceous	—	—	— —	carotene(s)	5 to 7	I II III	502 to 505 (acidic) 506 to 510 (hot KOH →acid) 514 to 520; 483 to 485	mytiloxanthin astaxanthin→ astacene zeaxanthin
Pisaster giganteus	6	(combined extract)	510; 483 519; 484	zeaxanthin *ester* β-carotene	5 to 7	I II III	516; 482 505; (495 in pyridine) (hot KOH →acid with same spectra) 513 to 518; 485 to 486	antheraxanthin metridene zeaxanthin
Ophiopteris papillosa	5	II III	497; — 505; — whole; 502; 471	esters taraxanthin *ester*	4	I II III IV	501; 470 503; 468 506 (not rendered acidic by hot KOH) 522; 489	taraxanthin a new xanthophyll? pectenoxanthin
Ophiothrix spiculata	11 or more	IX X XI	466 475 499; 466; 434	new acid on hydrolysis new acid on hydrolysis new xanthophyll *ester*	5	I II	495; 467 505	new xanthophyll new xanthophyll
Ophiothrix rudis	5	II V	505 460 whole; 497; 466 (before hydrolysis)	new acid on hydrolysis new acid on hydrolysis xanthophyll esters	more abundant than epiphasic fraction, but decomposed too rapidly for analysis			

like those encountered in the asteroids) but migrated quantitatively from this solvent to 90 per cent methanol in the partition test. It was adsorbed from petroleum ether solution at the top of a calcium carbonate column and was eluted therefrom with difficulty by methanol and acetic acid. Its solution in absolute ethanol gave a single band in the red at 663 mμ.

Ophiothrix spiculata: A yellow-green pigment remained in the diluted and salt-containing acetone extract from which all carotenoids had been transferred to petroleum ether. Insoluble in the latter solvent, even in the presence of dilute acids, this pigment was readily extracted with diethyl ether and transferred to absolute ethanol, in which it showed a single absorption band at 663 mμ.

Ophiothrix rudis yielded small quantities of a green pigment similar in solubility properties to that recovered from *O. spiculata.*

MacMunn's green pigments extracted from the radial caeca of *Solaster* and *Uraster* displayed several spectral bands in alcohol solution, one in the vicinity of a single band found in the red by ourselves, and the others in additional regions similar to those shown for chlorophyll. He considered that such green pigments were breakdown products of chlorophyll (MacMunn, 1886).

We have at present no new suggestions to add to MacMunn's conclusions. While the spectra of our green pigments were far simpler than those of MacMunn or than those of chlorophyll, it is likely that these pigments in the digestive organs of carnivores represent porphyrins from an *original* source of chlorophyll.

DISCUSSION

The major results obtained in this study are summarized in Table I. The animals are grouped according to their food habits. The echinoids feed on kelp, on detritus that is predominantly of vegetable orgin, or upon fixed algae, while the holothurians are bottom dwellers that subsist on the latter two classes of food. The asteroids feed exclusively on animal matter, especially mollusks, while the ophiuroids feed mainly on very small particulate animal matter.

A few calculations based upon information given in Table I reveal some interesting qualitative and quantitative differences between the carotenoids of herbivores and those of carnivores. For example, a total weight of 847 grams of tissue from herbivorous forms yielded some 4.27 mg. of carotenoid pigment (β-carotene equivalents), or an average concentration of about 0.50 mg. per 100 grams of fresh tissue. In the carnivores, on the other hand, 916 grams of tissue yielded a measurable quantity of some 14.61 mg. of carotenoids (β-carotene equivalents), giving an average concentration of about 1.60 mg. per 100 grams of fresh tissue. The average ratios of hypophasic to epiphasic pigments were, in the herbivores, 0.86, and in the carnivores, at least 13. Furthermore, account is to be taken of the fact that the hypophasic pigments in the ophiuroids were, as in the other carnivores, far in excess of the epiphasic ones, but that, in three out of four analyses, the color of the hypophasic fraction bleached before quantitative estimations could be made. If we assume that the hypophasic pigments in the second catch of *Ophiopteris papillosa* exceeded the epiphasic fraction by the same ratio as was found in the first catch (i.e. about 6) and that this ratio could be applied also to the other two ophiuroid species, *Ophiothrix spiculata* and *O. rudis,* we then arrive at a figure of 2 mg. of carotenoids per 100 grams of fresh carnivore tissue.

Among the carnivores, the ophiuroids contained oxygenated carotenoids exclusively, the epiphasic fractions yielding no carotenes, but only esterified xanthophylls and acidic carotenoids. The asteroids likewise showed a great preponderance of oxygenated over hydrocarbon carotenoids. *Pisaster giganteus* contained an epiphasic esterified xanthophyll which behaved like zeaxanthin. In the herbivores, the echinoids too contained some xanthophyllic pigments without exception, and most of them yielded an oxygenated carotenoid, echinenone, in the epiphasic fraction. One of three catches of *Strongylocentrotus purpuratus* yielded an esterified xanthophyll resembling lutein.

In summary, carnivorous species contained three to four-fold the quantity of carotenoid pigments found in herbivores. The oxygenated type of carotenoid, including xanthophylls and acidic compounds, preponderated vastly over carotenes [2] in carnivorous species. However, the herbivores, with the exception of the urchin *Strongylocentrotus purpuratus* which showed a slight excess of xanthophylls over carotenes, possessed predominating quantities of the latter type of pigment. Finally, the non-hydrocarbon carotenoids stored in carnivorous animals were more heavily oxygenated than those in the herbivores, the former yielding numerous carotenoid acids, as well as taraxanthin and similar xanthophylls, none of which were encountered in any of the herbivores.

The apparently greater capacity of the carnivorous, as compared with the herbivorous echinoderms, to assimilate or store carotenoids, with perhaps a certain degree of oxidative modification, may be associated with less metabolic utilization of the oxidized pigments than of the carotenes. At present nothing seems to be known either of the requirements of marine invertebrates for vitamin A or its precursors, or of specific rôles played by carotenoids in lower phyla.

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[2] It should be understood that the term "carotenes" as used here means those epiphasic pigments resistant to alkaline hydrolysis, and hence may include such mono-hydroxy xanthophylls as cryptoxanthin and the ketone echinenone. The possible presence of cryptoxanthin was usually eliminated, however, by employing in the partition test 95 per cent methanol, in which this pigment is preferentially soluble.

The carotenoid acids are of especial interest and may be classified into three types. *In vivo* some are neutral xanthophylls but treatment with alcoholic alkali produces molecular rearrangements and oxidations which lead to the formation of acids (*cf.* astaxanthin). This type was found only in the asteroids but was present in all such specimens examined. The second type is acidic as it is extracted from the tissues. It was found only in *Pisaster,* and since it resembles strongly the mytiloxanthin described by Scheer (1940) from the California mussel, it is likely that *Pisaster* derives this pigment from the mollusk, upon which it feeds extensively. Finally, acids may occur *in vivo* as esters, in which case they are epiphasic before hydrolysis, their acidic properties becoming evident after treatment with alcoholic potash. These esters were found only in ophiurans and gave on hydrolysis a new type of pigment, which is acidic, and has a single absorption maximum at 460, 466 or 475 mμ. In addition, an acid-yielding ester of the more conventional type with a single absorption maximum at 505 mμ appeared.

As in the lobster, carotenoid acids were found to be combined with protein in one case. *Pisaster giganteus* contains purple, blue and yellow chromoproteins in the skin, the purple and blue patches of which yielded an unesterified carotenoid, which formed an acid on treatment with alkali. This pigment resembled the anemone pigment metridene (Fox and Pantin, 1941).

Table II shows some similarities between certain of the echinoderm carotenoids and known pigments. A commonly occurring one showed a spectrum like that of β-carotene or zeaxanthin; pigments in this group were found in all classes except the Ophiuroidea, which yielded instead pigments with spectra like taraxanthin, not found in the other classes. Spectra like that of α-carotene or lutein were not frequently encountered, except among the echinoids, wherein both carotenes and xanthophylls with this type of spectrum were found. Pigments resembling echinenone have been mentioned above, notably in the epiphase of echinoid extracts (Lederer, 1938). The unchromatographed extracts, both epiphasic and hypophasic, of *Stichopus* also showed the echinenone type of spectrum; again the alkali-resistant epiphase and the hypophase of one of the carnivores, i.e., *Astropecten,* as well as certain hypophasic pigments (rare xanthophylls such as pectenoxanthin) in other groups, manifested the same absorption maxima.

Where individual tissues were studied, the highest values were found in the digestive tract, excepting in the testes of *Lytechinus;* certain asteroid skins would also show relatively high values were they readily separable from the colorless skeleton.

The few correlations which could be observed between sex and carotenoid content in tissues were limited to three of the echinoid species. In *Dendraster,* males contained about twice the concentration of epiphasic and thrice the hypophasic pigments found in females. Estimations of pigments in individual tissues were not made. In *Strongylocentrotus purpuratus,* the skin of each sex yielded only traces of carotenoids. In the intestine, epiphasic pigments were plentiful and of the same order of concentration in both sexes; the same was true of the hypophasic compounds. However, carotenoids in ovaries exceeded those in testes by three-fold, and were entirely epiphasic, xanthophylls being absent. In *Lytechinus,* whole males yielded somewhat more of each class of carotenoid than did whole females. This was due to a five-fold excess of epiphasic and nearly a two-fold excess of hypophasic carotenoids in testicular over ovarian tissues. Intestines, considerably lighter in mass than gonad material, were also fairly rich in pigment, females yielding nearly a third again as much epiphasic and almost three times as much hypophasic pigment as males. The skin of *Lytechinus,* like that of *Strongylocentrotus,* contained only traces of carotenoids.

Females of *Strongylocentrotus* and *Lytechinus* contained some 25 and 27 per cent, respectively, of their carotenoids in the ovaries, whereas corresponding males mobilized approximately 17 and 77 per cent, respectively, to their testes, although the sperm itself was not colored.

The carotenoid distribution ratio between intestine and ovary is doubtless subject to variation with discharge of ripe eggs. There still remains the striking mobilization of carotenoids to testicular tissues, especially in *Lytechinus.* Also remarkable was the complete absence of xanthophylls from the gonads of both sexes of *Strongylocentrotus.*

Three types of non-carotenoid pigment were encountered in this study. Insoluble pigments, presumably of the melanin type, appeared in *Stichopus* and *Pisaster ochraceus,* but were not studied. Green pigments, the " enterochlorophylls " of MacMunn, were obtained from certain of the carnivores, but were never found in starved herbivores. These pigments invariably had absorption maxima at 661 and 663 mμ in absolute ethanol. From all the echinoids, but from no others, much red to purple pigment of the echinchrome type was extracted. It was found as calcium salt in the testes and spines, and as purple (basic) or red (acidic) irregular bodies dispersed in the tissues of ectoderm, endoderm, intestine, and in some cases, in the gonads. Although quantitative studies were not made, echinochrome was clearly most abundant in *Strongylocentrotus,* quite abundant in *Dendraster,* and present in relatively small amounts in *Lytechinus.*

In conclusion it may be reiterated that the echinoderms represent a structurally and ecologically diverse group of much interest for further investigations of a comparative biochemical nature, especially regarding the metabolism of colored compounds.

SUMMARY

1. The pigments of echinoderms, belonging to twelve species, nine genera and four classes, have been studied qualitatively and in part quantitatively, with the aid of certain standard methods.

2. Carnivorous species contained more carotenoids in the aggregate than did herbivores, by some three or four-fold.

3. Among the echinoids, *Dendraster* and *Lytechinus* males yielded more total carotenoids than did females, while in *Strongylocentrotus purpuratus,* the concentrations in each sex were similar. Females of *Strongylocentrotus* and *Lytechinus* contained about a fourth of their carotenoids in the ovaries, whereas corresponding males mobilized about one-sixth and three-quarters, respectively, to their testes.

4. Oxygenated carotenoids, including xanthophylls and acidic compounds, preponderated vastly over the hydrocarbon type (carotenes) in carnivores, while in most herbivores epiphasic pigments, including carotenes and echinenone, showed some degree of predominance.

5. The presence of the ketonic carotenoid echinenone was indicated in most of the echinoids; its presence was also regarded as likely in another member of the herbivore-omnivore group, i.e., the cucumber *Stichopus,* and in one carnivore, *Astropecten.*

6. Carotenoid acids, or compounds which yield carotenoid acids on treatment with alkali, were found only in carnivorous species. A new type of epiphasic pigment, with a single absorption maximum in the violet at values of from 460 to 475 mμ, yielding an acid on hydrolysis, was found in the ophiuroids.

7. Xanthophyll esters were found consistently in the Ophiuroidea and in one of the Asteroidea, *Pisaster giganteus.* In the ophiuroids, they were of the heavily oxygenated type, and replaced carotenes, which were completely lacking. One of three catches of *Strongylocentrotus purpuratus* yielded some esterified lutein-like xanthophyll.

8. The occurrence of echinochromes, found only in the echinoids, and of green pigments, "enterochlorophylls," found in the intestines of carnivores, is discussed.

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